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COMPARISON OF THE EFFECT OF HYPOPHYSECTOMY AND THYROXINE ADMINISTRATION ON THYROID FUNCTION IN THE RAT[1]

TAKASHI YAMADA,[2] SHIRO IINO[2] AND MONTE A. GREER

Division of Endocrinology, Department of Medicine, University of Oregon Medical School, Portland, Oregon

ABSTRACT

Comparison was made of the changes in various thyroid parameters at different intervals following thyroxine administration and hypophysectomy in 332 adult male rats. There was a distinct temporal difference among the responses of the different parameters to the acute fall in thyrotropin levels induced by thyroxine administration or hypophysectomy. The most sensitive parameter was thyroidal release of radioiodine, the next I^{131} uptake and the least sensitive, thyroid weight. Hypophysectomy and thyroxine injection produced nearly identical effects on thyroidal I^{131} uptake and release. After thyroxine administration a rebound phenomenon was observed in both I^{131} uptake and in thyroid weight, but it was not observed in I^{131} release. Potassium iodide, in amounts equivalent to the doses of thyroxine, decreased thyroidal I^{131} for a short period but had little effect on thyroid weight. Sham hypophysectomy resulted in decreased I^{131} loss from the thyroid, the degree of thyroidal suppression depending upon the extent of surgically induced pituitary trauma.

IT IS well established that the regulation of thyroid function is almost wholly dependent upon the secretion of thyrotropin from the pituitary gland (1–5). The administration of exogenous thyroid hormone causes a decrease in the secretion of TSH (4, 5). Conversely, a decrease in circulating thyroid hormone, such as that produced by chronic antithyroid drug administration, results in augmented TSH secretion (1, 6, 7). The mechanism acting to thus maintain a constant level of thyroid hormone in the blood is similar to other so-called "servo" mechanisms (2, 8).

It is possible that factors other than the circulating thyroxine level itself may modulate TSH secretion. Goldberg *et al.* (17), for example, found that dinitrophenol administration as well as other experimental conditions which increase body temperature would produce decreased TSH release

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[2] Trainee of National Institute of Arithritis and Metabolic Diseases.

associated with a fall in protein-bound iodine. The demonstrated suppression of TSH release by systemically ineffective amounts of thyroxine injected directly into the hypothalamus or pituitary (13, 14) indicates that the concentration of thyroid hormone reaching the receptor area must still be considered an important regulatory mechanism, however. The level of free thyroxine, rather than that of bound hormone as reflected by the PBI, is probably the controlling factor (19).

There has been some controversy over whether the suppression of thyroid function caused by thyroxine is mediated entirely through a decrease in hypophyseal TSH secretion (9, 10), or whether there is in part a direct inhibitory action of thyroxine on the thyroid gland (11, 12). Many previous studies have been made of the effects of hypophysectomy and thyroxine administration on thyroid function, e.g. (3, 5, 13, 18, 20, 21, 22), but a detailed comparison of the changes produced in various thyroid parameters at different intervals following thyroxine administration or hypophysectomy has not been reported.

We therefore considered it of interest to compare the effect of these two procedures on thyroid weight, thyroidal radioiodine uptake, and thyroid hormone secretion.

MATERIALS AND METHODS

Adult male Holtzman rats were employed. Animals in which thyroid secretion was measured weighed 200 to 250 grams; all others weighed 100 to 130 grams. All the rats were fed a Remington low iodine diet beginning seven days before the experiments to permit an adequate accumulation of radioiodine by the thyroid. Radioiodine uptake was studied by injecting 0.1 μC. of carrier-free I^{131} intraperitoneally, sacrificing the animals four hours later, and weighing the thyroids on a torsion balance before counting them in a well-type scintillation counter.

Thyroid hormone secretion was measured as follows: 20 μC. of I^{131} were injected intraperitoneally; beginning 24 hours later, 30 mg. of propylthiouracil in 10% gum acacia were given once daily subcutaneously at rotated sites to block the reaccumulation in the thyroid of I^{131} liberated from degradation of endogenously labeled thyroxine. Counts over the thyroid region were made five times daily with an externally placed scintillation counter using light ether anesthesia. Each count was expressed as a percent of the initial thyroidal uptake on semilog paper. Although the decline in thyroidal radioactivity thus measured may reflect a loss of labeled compounds in addition to thyroxine and triiodothyronine, it still provides a useful method of assessing the rate of thyroidal secretion. After a straight line of more than five individual points had been obtained, L-thyroxine dissolved in 0.5 ml. physiological saline was administered intraperitoneally in varying concentrations as either single or repeated injections.

Hypophysectomy was performed by the parapharyngeal route. Completeness of hypophysectomy was checked at autopsy by examination of the sellar region under a 10 × dissecting scope. All animals with pituitary remnants except those in the sham hypophysectomy group were discarded. The extent of pituitary damage produced by sham hypophysectomy was studied by fixing the unroofed skull with the brain intact in 10% formalin and subsequently making serial sections through the pituitary region.

The degree of pituitary cell damage and of bleeding into the pituitary were separately assessed in each sham hypophysectomy animal. The degree and duration of inhibition

TABLE 1. THYROIDAL I^{131}-RELEASE AFTER HYPOPHYSECTOMY OR THYROXINE INJECTION

Treatment	No. of animals	T 1/2* before op. or inj. (hours)	T 1/2 during inhibition (hours)	Time of onset of inhibition (hours)	Duration of inhibition (hours)	Duration of inhibition after last injection (hours)
Hypophysectomy	7	18.9 (±2.3)[1]	310.0 (±35.0)	1.7 (±0.5)	—	—
Thyroxine Inj. (once daily)						
10γ ×1	4	16.8 (±2.9)	90.5 (±10.0)	1.8 (±0.6)	67.5 (±15.0)	—
20γ ×1	5	13.5 (±0.4)	107.5 (± 8.7)	1.5 (±0.2)	57.5 (± 9.3)	—
10γ ×3	4	20.5 (±3.0)	140.3 (±15.5)	1.4 (±0.1)	117.3 (±14.6)	80.0 (±11.3)
10γ ×7	3	18.7 (±1.0)	182.0 (±14.0)	1.8 (±1.0)	208.0 (± 8.7)	75.0 (± 5.0)

* T 1/2: Biological Half Life.
[1] Mean and Standard Error.

of thyroidal secretion were similarly separately assessed. All four parameters were graded from 0 to +++ and the two indices of pituitary damage totaled as well as the two indices of thyroidal suppression. These values were then plotted for each animal to correlate the degree of thyroidal suppression with that of pituitary damage.

RESULTS

The effect of hypophysectomy and thyroxine injection on thyroidal radioiodine uptake

After hypophysectomy radioiodine uptake decreased rapidly during the first 48 hours and reached a plateau by the fourth postoperative day (Fig. 1). A very similar pattern was produced by the daily injection of 5 μg. of thyroxine. The minimum values observed on the seventh day were almost the same in both groups.

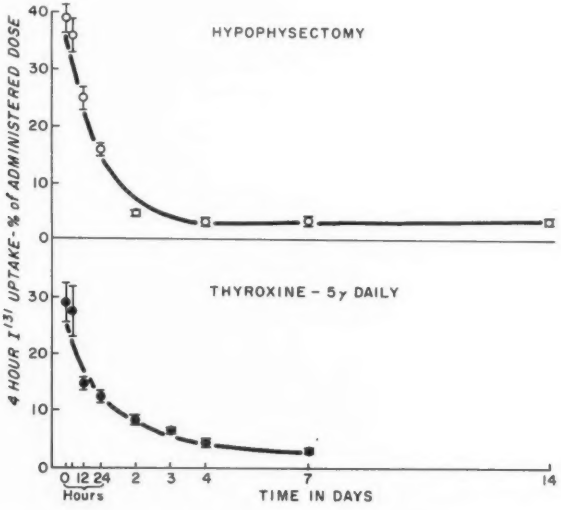

FIG. 1. Comparison of thyroidal I^{131} uptake after hypophysectomy or the daily administration of thyroxine. Each point and vertical bar represent the mean and standard error for five to eight animals.

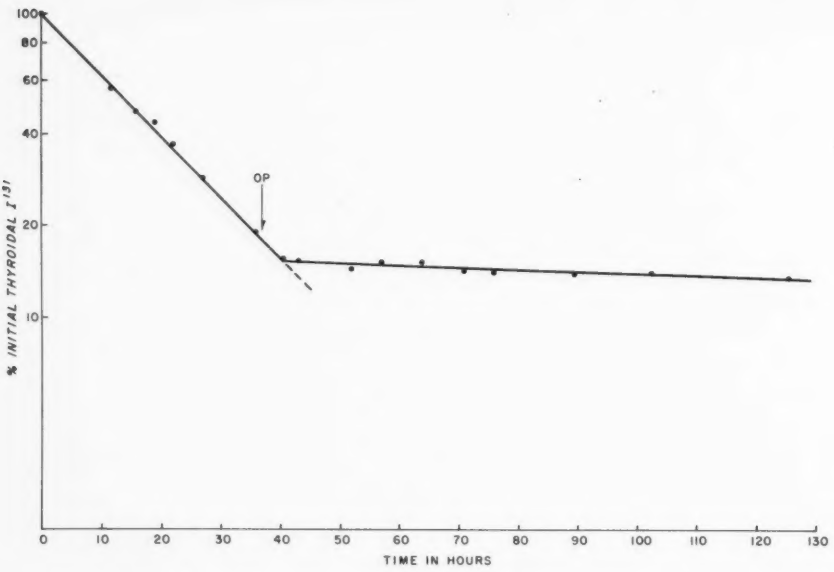

FIG. 2. Thyroidal I^{131} release after hypophysectomy.

The effect of hypophysectomy and thyroxine administration on thyroid hormone secretion

The results are summarized in Table 1. The biological half-life of the thyroidal radioiodine averaged 18.9 hours before hypophysectomy and 310 hours postoperatively, indicating that pituitary ablation produced a marked suppression of thyroid secretion (Fig. 2). The average latent period before the onset of inhibition was 1.7 hours.

Twenty-three animals were subjected to "sham hypophysectomy," which involved several increasingly traumatic approaches to the pituitary gland without actually removing it. In Group 1 only the outer table of the basisphenoid bone was removed with the trephine. In Group 2 both tables were removed, exposing the pituitary capsule. In Group 3 the pituitary capsule was cut without attempting to damage the hypophysis itself. In Group 4 a hooked needle was twisted in the substance of the pituitary gland to produce hypophyseal damage. The biological half-life of thyroidal radioiodine preoperatively in these animals averaged 18.4 hours. The majority of the animals in the last three sham hypophysectomy groups showed diminished thyroid hormone secretion (Fig. 3). Only when surgery was limited to removing the outer table of bone was no effect produced. The change usually appeared within 5 to 12 hours postoperatively and lasted for approximately one day. Histological study revealed that all animals showing decreased secretion had varying degrees of pituitary

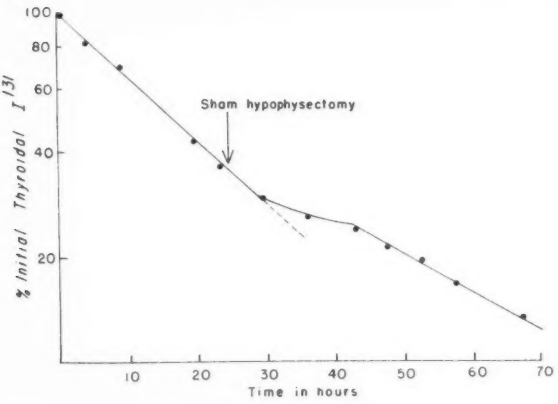

FIG. 3. Four hours after opening the pituitary capsule in a sham hypophysectomy a slight suppression of thyroidal secretion appeared which lasted for about 15 hours

damage, mainly in the medial portion of the adenohypophysis. The extent of pituitary damage could not be correctly assessed by the amount of hypophysial trauma estimated at the time of operation to have been produced surgically. The results are summarized in Figure 4.

To compare the effect of exogenous thyroxine with hypophysectomy, rats were injected with graded doses of T4 for different periods of time. As seen in Table 1, the initial biological half-life was similar to that of the hypophysectomy group. The latent period following thyroxine injection before thyroid secretion began to decrease (Fig. 5) was indistinguishable from that following hypophysectomy. However, the decline in secretion produced by 10 or 20 μg of thyroxine was not nearly as complete as that following hypophysectomy. The duration of inhibition following a single injection of thyroxine was the same whether 10 or 20 μg were given. Al-

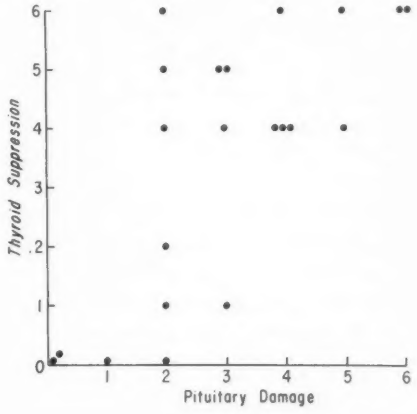

FIG. 4. Correlation of thyroidal suppression with pituitary damage in sham hypophysectomized rats. The correlation coefficient is 0.724.

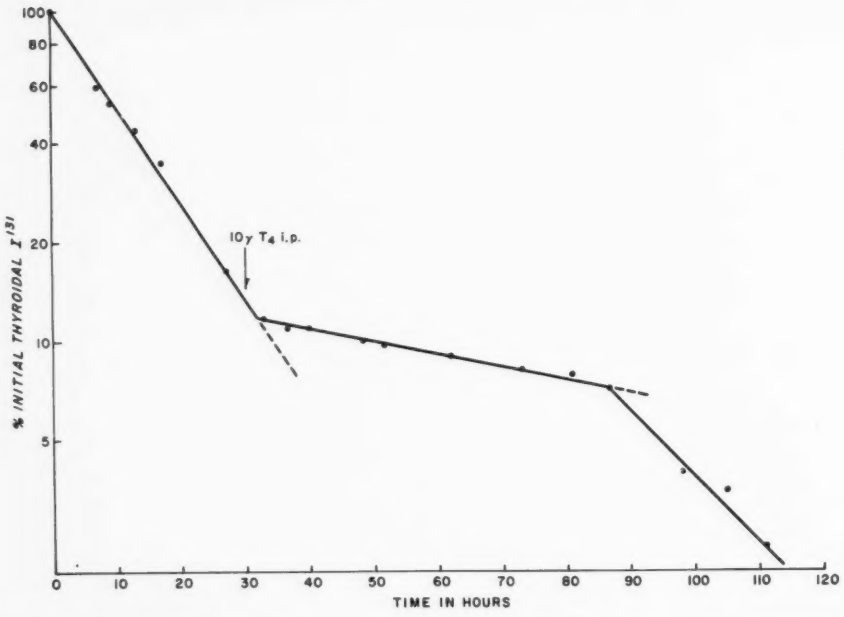

FIG. 5. Three hours after the intraperitoneal injection of 10 μg L-thyroxine, release of thyroidal I^{131} was suppressed for 54 hours.

though repeated daily injections of thyroxine caused a period of inhibition (after the final injection) slightly longer than that following a single injection, there was no significant difference between the animals injected for 3 days and those injected for 7 days.

The effect of a single injection of thyroxine on thyroidal iodine uptake and thyroid weight

The effects of a single injection of either 5 or 10 μg of T4 on thyroidal radioiodine uptake were studied at the intervals shown for the first experiment in Figure 6. Five to eight animals were used for each point. A pronounced suppression, with the nadir at 48 hours, was produced by both doses. This was followed by a fairly rapid recovery and a return to control levels by the fourth day. Since the uptake was approximately the same on the seventh day as on the fourth, we wondered whether it remained at this level after the fourth day or whether there was a rebound phenomenon characteristic of sevo reactions. The later proved to be the case when the experiment was repeated and the uptake measured on days four through eight. As shown in Figure 6, on the fifth day the uptake was almost double the control value. It reached its acme at 292% of the control level on the sixth day.

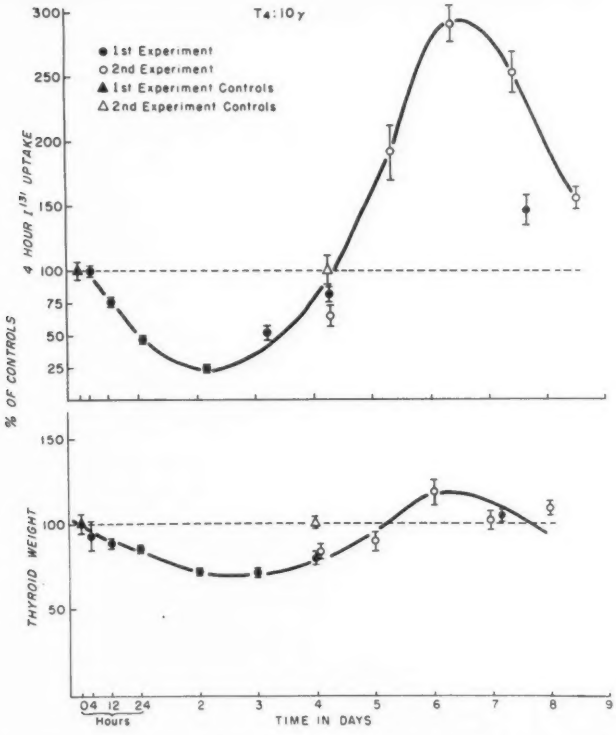

FIG. 6. Graphic combination of the first and second experiments studying the effect of a single 10 μg dose of L-thyroxine. Each point represents five to eight animals. The results are expressed as percent of the untreated controls run with each experiment since they were performed at different times. A study with a single injection of 5 μg T4 parallel to the first experiment depicted here gave identical results.

The changes in thyroid weight after a single injection of 10 μg T4 are also depicted in Figure 6. Although the pattern was similar to the one observed with radioiodine uptake, the amplitude of fluctuation was much smaller. The minimum was reached on the third day and the maximum on the sixth day.

The suppression of radioiodine uptake produced by a single injection of 150 μg of T4 (Fig. 7) was considerably more pronounced than that after 5 or 10 μg. This seemed to be due primarily to the longer duration of action of the larger dose, since the curves for the first two days were identical with all doses. A minimum uptake was observed four days after injection compared to a minimum at two days with the smaller doses. Thyroid weight also reached a minimum on the fourth day. Neither thyroid weight nor radioiodine uptake had returned to normal by the seventh day.

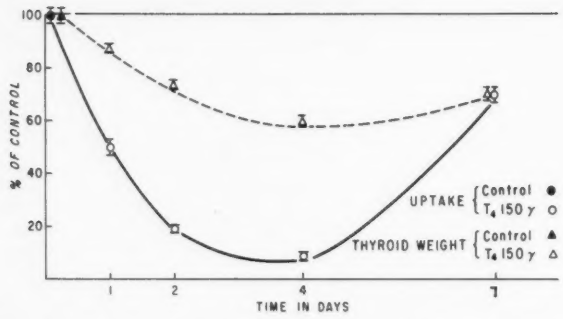

FIG. 7. Thyroid weight and radioiodine uptake after a single injection of 150 μg L-thyroxine. Each point represents the mean and standard error of five to eight animals.

The effect of iodide on thyroidal weight and radioiodine uptake

After a single injection of 8.3 μg KI (equivalent in iodine content to 10 μg T4), thyroidal radioiodine uptake was markedly reduced at four hours but returned to normal by the second day (Fig. 8). There was a very slight decrease in thyroid weight at 24 hours. On the other hand, 128 μg KI (corresponding to 150 μg T4) greatly suppressed radioiodine uptake for the first 24 hours (Fig. 9). The uptake did not return to normal until the seventh day. Thyroid weight after the larger dose of KI showed a slight continuous decrease throughout the period of observation.

DISCUSSION

The data presented above indicate that hypophysectomy and repeated daily injections of physiological doses of thyroxine produce very similar effects on the thyroid gland. Suppression of radioiodine uptake proceeded along an almost identical pattern in both groups and no greater decrease occurred in the thyroxine-treated than in the hypophysectomized rats. It

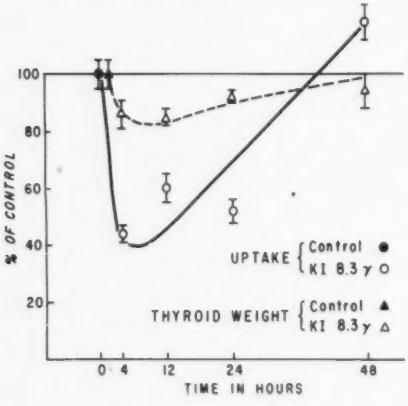

FIG. 8. Thyroidal I^{131} uptake and weight after the single injection of a small dose of potassium iodide corresponding in iodine content to 10 μg thyroxine. Each point represents 5 to 8 animals.

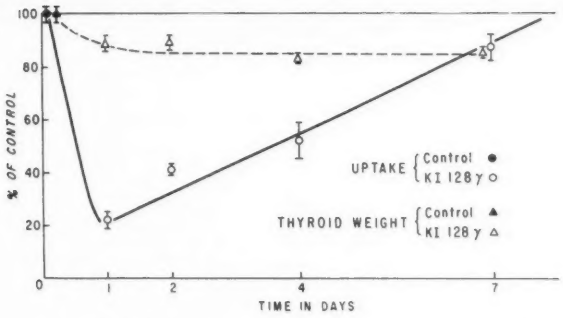

FIG. 9. Thyroidal radioiodine uptake and thyroid weight after the single injection of a large dose of potassium iodide, corresponding in iodine content to 150 μg thyroxine. Each point represents 6 to 8 animals.

thus appears that thyroxine may suppress thyroid function solely by inhibiting secretion of thyrotropin from the pituitary and that it is not necessary to postulate a direct inhibitory action of thyroxine in the thyroid gland itself. Such an hypothesis is consistent with the further observation that intrahypophyseal injections of systemically ineffective amounts of thyroxine produce thyroidal suppression indistinguishable from that of effective systemic doses (13, 14). The observation of Halmi *et al.* (23) that large doses of triiodothyronine will suppress the thyroid/serum (T/S) iodide ratio to lower levels than will hypophysectomy suggests that there may be some direct inhibitory action of thyroid hormones on the thyroid gland. However, the significance of their findings is not yet certain since they found that T3 did not produce the same suppression of the T/S ratio in hypophysectomized as in intact rats, a "permissive" action of the pituitary being implied.

It is of interest that thyroidal secretion was not depressed to a level comparable to that which followed hypophysectomy by doses of thyroxine which were fully effective in suppressing iodine uptake and thyroid weight. This observation is in agreement with the previous data reported by Astwood and Bissell (15) that the amount of T4 required to prevent goiter in rats chronically treated with antithyroid drugs will not prevent thyroidal iodine depletion.

There is almost unanimous concensus that the commonly utilized thyroid parameters are not equally sensitive to a given level of thyrotropin. In order of increasing sensitivity to TSH, they are: (1) thyroid weight and histology, (2) radioiodine uptake, and (3) thyroid hormone secretion. This variation in sensitivity was also demonstrated in the present study (Fig. 10).

All three parameters began to decrease at approximately the same time following hypophysectomy or thyroxine administration, although during

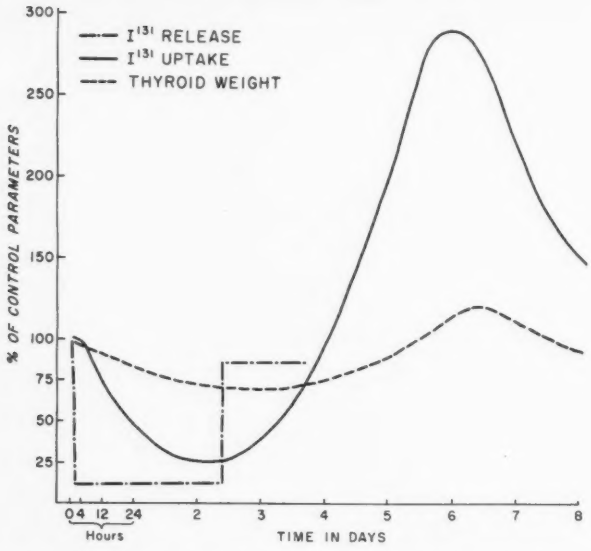

FIG. 10. Comparison of changes produced in different parameters of thyroid activity after a single physiologic dose of thyroxine.

the first 12 hours it was rather difficult to find a statistically significant decrease in thyroid weight. During the recovery phase from the change induced by a single dose of thyroxine, however, there was a certain temporal difference among the three functions. Thyroid hormone secretion was the most sensitive, returning most rapidly to normal; thyroid weight was the least sensitive, and radioiodine uptake was intermediate between the two. Such changes have indicated to some investigators that it is thyroid secretion that is primarily affected by TSH and that the other changes occur secondarily (16). However, it is possible that the individual enzyme systems responsible for each metabolic function of the thyroid gland have their specific optimal TSH requirement during which a proportional response to varying concentrations is exhibited. Before this level is reached, or once it has been exceeded, there would be very little increase of activity with increasing amounts of TSH. Thus the augmented quantity of TSH coming from the adenohypophysis during immediate recovery from suppression induced by a single dose of thyroxine might be large enough to produce near maximal stimulation of thyroid secretion. A much longer time lag might ensue before sufficient TSH were secreted to produce a maximal radioiodine uptake. This might also explain why radioiodine uptake rose so far above normal between the fourth and the seventh days, while thyroid secretion, as far as we were able to determine, stayed at the normal level. Release of thyroid hormone, having already been maximally stimulated, would not respond further to the increased amounts of thyro-

tropin being secreted in the later phase of recovery while the iodine concentration and binding mechanisms would require these higher levels of TSH for optimal activity.

Unfortunately, because of the rapid loss of thyroidal radioiodine during the control period in the animals in which thyroid secretion was studied, insufficient thyroidal radioactivity remained to continue following the secretion rate to the time when the maximal rebound was observed with radioiodine uptake. In addition, propylthiouracil was administered to the secretion rate rats but not to the other animals. It is not known whether this dissimilarity in treatment also might explain the difference observed in the two functions.

It has long been postulated that iodide may suppress thyroid activity by inhibiting TSH secretion from the pituitary or by interfering directly with TSH activation of the thyroid gland. These possibilities should be considered in this investigation, particularly where a large amount of thyroxine was used, since the relative amount of iodide accumulation in the thyroid from the degradation of administered thyroxine could be considerable in these iodine-deficient rats. However, these mechanisms cannot explain our results since potassium iodide suppressed thyroidal I^{131} uptake for only a short period without any appreciable decrease in thyroid weight, contrasting sharply with the thyroxine experiments. The observed effects of iodide in our experiments seem best explained by a sudden great increase in the iodide pool of the body, rather than any specific inhibition or inactivation of TSH. The effect of iodide on thyroidal secretion was not studied here, but Wolff had previously found that iodide did not appreciably decrease the rate of loss of thyroidal I^{131} under similar conditions (22).

The prompt decrease in thyroidal secretion following sham hypophysectomy was unexpected. Randall and Albert (21) did not observe any change in thyroidal radioiodine metabolism after sham hypophysectomy in the rat, but the extent of their operative approach was not recorded. Our histologic studies indicate that the extent of pituitary trauma probably determined the degree of decreased TSH release postoperatively. We anticipated that pituitary mutilation would, if anything, accelerate rather than decrease the acute release of thyrotropin since we expected preformed TSH from the damaged cells to enter the blood stream more readily. Even very minor pituitary damage in some instances resulted in quite a profound decrease in thyroidal secretion. Since the initial inhibition of thyroidal activity in the sham hypophysectomy group usually appeared 5–12 hours postoperatively compared to 1–2 hours after hypophysectomy or thyroxine administration, it is possible that secretion of preformed thyrotropin may have continued relatively unimpaired but that synthesis of TSH was decreased by surgically induced circulatory embarrassment. It is unlikely that non-specific pituitary-adrenal activation was responsible since decreased thyroidal secretion was not produced by those operations which

did not expose the hypophysial fossa. A completely satisfactory explanation must await further study.

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EFFECT OF TECHNICAL PERTHANE FRACTIONS ON DOG ADRENAL CORTEX[1,2]

MARVIN J. BLEIBERG

Department of Pharmacology, Jefferson Medical College, Philadelphia, Pennsylvania

ABSTRACT

A method for the chemical fractionation of perthane and the isolation of relatively pure o,p′ isomer is described. P,p′ isomer, and crude mixtures of isomers were administered orally for one to eight weeks to dogs. Pups similarly received either the p,p′ or the o,p′ isomer.

Recrystallized p,p′ isomer or crude fractions of perthane containing a mixture of isomers produced moderate to marked adrenocorticolytic atrophy. Relatively pure o,p′ perthane was somewhat less active than the p,p′ isomer in the pup. The polar by-products added no significant adrenal toxic effect to the crude oily mixture. The total adrenocorticolytic activity of technical perthane in the dog was attributable to the additive effects of its isomers.

PERTHANE (2,2 bis(p-ethylphenyl)-1,1-dichloroethane), a commercial insecticide analogous to DDT has been shown on chronic oral administration to dogs to produce adrenal cortical atrophy (1–3) resembling that induced by DDD (4). The principal component of the technical preparation of these agents is the p,p′ isomer (Fig. 1). The o,p′ isomer is the principal contaminant. It has been suggested that the active adrenocorticolytic principle of DDD is the o,p′ isomer (5, 6). This was based on the observations that the p,p′ isomer isolated from the technical product is inactive after several recrystallizations; moreover, the o,p′ isomer was more potent than the technical material. The potency of the technical material was attributable to its content of o,p′ isomer.

It was believed of interest to fractionate technical perthane for similar comparative studies of the adrenocorticolytic effect. This paper describes a procedure for the separation of these isomers from the technical product and studies of their effect on the canine adrenal cortex.

Fractionation of technical perthane. Two kilograms of technical perthane (lot #3468), a brownish waxy solid, were obtained from the Rohm and Haas Co. Five hundred gram lots were recrystallized from hot ethanol (one liter) and a white crystalline precipitate representing p,p′ perthane was obtained (m.p. 57–58° C uncorrected). The melting point of authentic p,p′ perthane is given as 56–57° C (7, 8). A brownish-colored oil was recovered from the ethanol and weighed 150 gm. (30% of the technical product). The

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[2] A portion of this work was presented at the meetings of the Federation of American Societies for Experimental Biology, Atlantic City, 1959 and an abstract appears in *Federation Proceedings* **18:** 369, 1959.

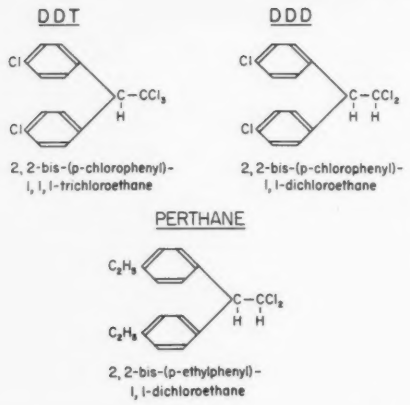


FIG. 1. Formulas of the p,p′ isomers of DDT, DDD and perthane for comparison.

p,p′ isomer was subjected to two further recrystallizations (m.p. 58–59° C). More p,p′ isomer crystallized from the brownish oil when allowed to stand in the refrigerator for several weeks. The resulting oil after removal of the crystalline material is designated residual oil I.

Paper chromatography. Paper chromatograms were prepared according to the reversed phase system of Winteringham (9). The spots were detected by the method of Mitchell (10). By these methods the recrystallized material gave a single spot representing the p,p′ isomer whereas the oil gave spots corresponding to those seen with known samples of p,p′ and o,p′ perthane.

Removal of polar by-products from residual oil. Twenty-five grams of the residual oil I was chromatographed on a column (45×260 mm.) of activated alumina. The fractions eluted with petroleum ether (1100 ml.) were pooled, and a clear colorless oil (II) obtained after evaporation of the solvent weighed 23.3 gm. (92%) of the initial oil. A paper chromatogram of an aliquot of the oil showed two spots, one of these corresponding to recrystallized p,p′ perthane, the other to the o,p′ isomer.

Dehydrochlorination. Ninety-eight grams of the residual oil I were subject to an alkaline dehydrochlorination procedure as described by Cristol (11) for a 24 hour period. On a paper chromatogram of the oily product obtained, the spot for p,p′ perthane, which was noted before alkali treatment, did not appear, but a spot corresponding in location to a known sample of dehydrochlorinated p,p′ perthane was now present. The spot for o,p′ perthane was again seen.

Oxidation. The oxidation of the dehydrochlorinated p,p′ perthane was conducted by the procedure of Scanlan and Swern (12). A yield of 48.6 gm. of a reddish-colored oil (III) was obtained. On a paper chromatogram of the oil a spot corresponding to the o,p′ isomer was detected and only a trace of the spot from the dehydrochlorinated p,p′ perthane.

Isolation of o,p′ perthane. The total yield of oil III was chromatographed on a column (45×250 mm.) of activated alumina. The first petroleum ether eluate (500 ml.) contained the relatively pure o,p′ isomer, a clear colorless oily product weighing 12.4 gm. A paper chromatogram of this oil showed a single spot at the expected location for o,p′ perthane. Subsequent fractions eluted with petroleum ether gave also a second spot, corresponding to the location of residual unoxidized dehydrochlorinated p,p′ material.

Properties of isolated o,p′ isomer. The product resisted crystallization and charred at about 250° C in an attempt to determine the boiling point. The refractive index at 28° C was 1.5760. An elemental analysis[3] of the isolated oil gave C = 69.27%, H = 6.35%, Cl = 23.99%. The corresponding values expected for a perthane isomer are

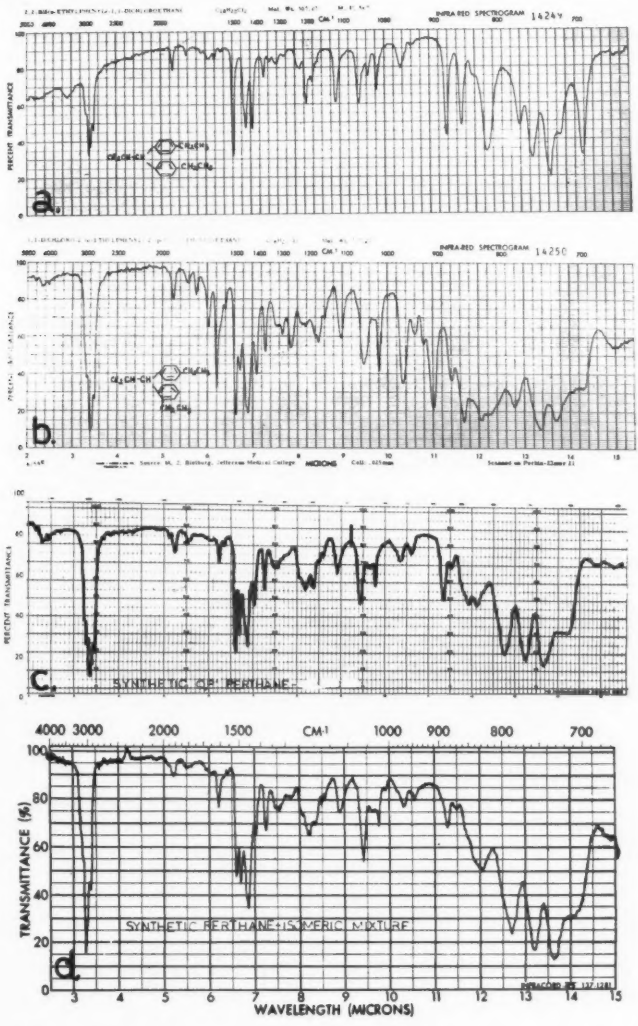


FIG. 2. Infra-red spectrograms in order from top to bottom: (a) p,p′ perthane, (b) relatively pure o,p′ perthane isolated by the author from technical perthane, (c) o,p′ perthane, m.p. = 49–50° C (synthesized by Nichols), and (d) isomeric mixture obtained by synthesis by the author. The spectrogram of p,p′ isomer is that of a very pure sample of p,p′ perthane. Peaks peculiar to the o,p′ isomer may be identified in the 735–860, 1090–1225, and 2000–1600 Cm.$^{-1}$ regions. These spectrograms of the o,p′ isomer isolated from the technical material, and of the mixture of isomers are not those of pure materials. The relatively pure o,p′ isomer isolated from technical perthane shows some additional absorption characteristic of contamination by an olefin (dehydrochlorinated p,p′ isomer) and by a bis-acetyl carbonyl compound (2,2 bis(p-acetylphenyl-1,1-dichloroethane). The spectrogram of the mixture obtained by synthesis shows bands indicative of a mixture of mostly o,p′ perthane with some p,p′ and some dehydrochlorinated material.

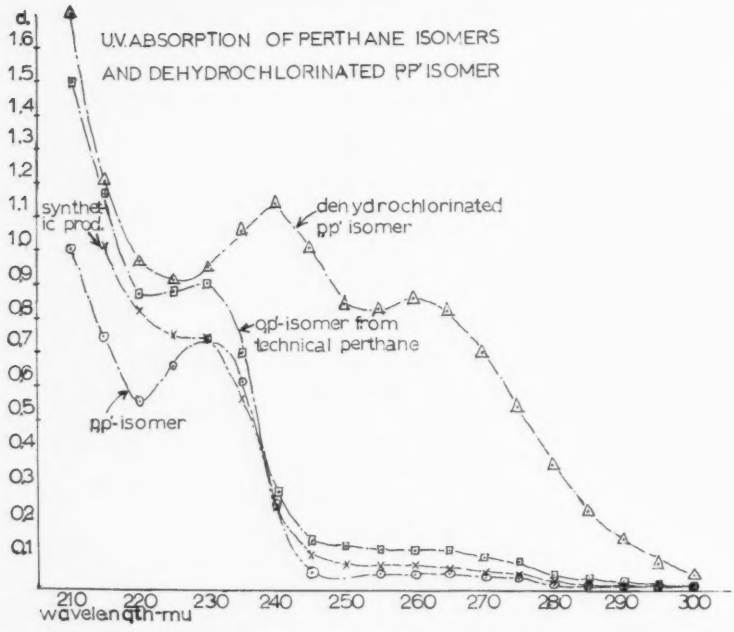


FIG. 3. Ultra-violet absorption spectra of p,p′ isomer, the synthetic isomeric mixture synthesized by the author, the o,p′ isomer isolated from technical perthane, and the olefin obtained by dehydrochlorination of p,p′ isomer. Note the extended range of ultra-violet absorption of the olefin and the olefin peak at 260 mu, and the limited range of absorption of the perthane isomers with a peak at 230 mu. (p,p′ isomer) or an inflection in the absorption curve at this wavelength.

C = 70.3%, H = 6.56%, Cl = 23.08%. The analysis indicated some contamination of the material with related products.

Synthesis of o,p′ isomer. Synthesis of the o,p′ isomer was attempted according to the method given by Haller *et al.* for o,p′ DDD (13) using the corresponding o-ethyl derivatives in place of o-chloro derivatives. Since one of the starting materials was a technical grade reagent, the final product, an oil similar to the o,p′ isomer isolated from technical perthane, proved to be a mixture of isomers when chromatographed on paper. Spots for both o,p′ and p,p′ isomer were detected. The refractive index of the oil at 28° C was 1.5782. The elemental analysis[3] of the oil was C = 67.75%, H = 6.09%, Cl = 25.79%. After the work reported in this paper was done, Nichols (14) prepared pure o,p′ isomer using Haller's method (13). The melting point of the four times recrystallized o,p′ isomer obtained by Nichols is 49–50° C. Elemental analysis of his product was C = 70.48%, H = 6.60%, Cl = 23.09%.

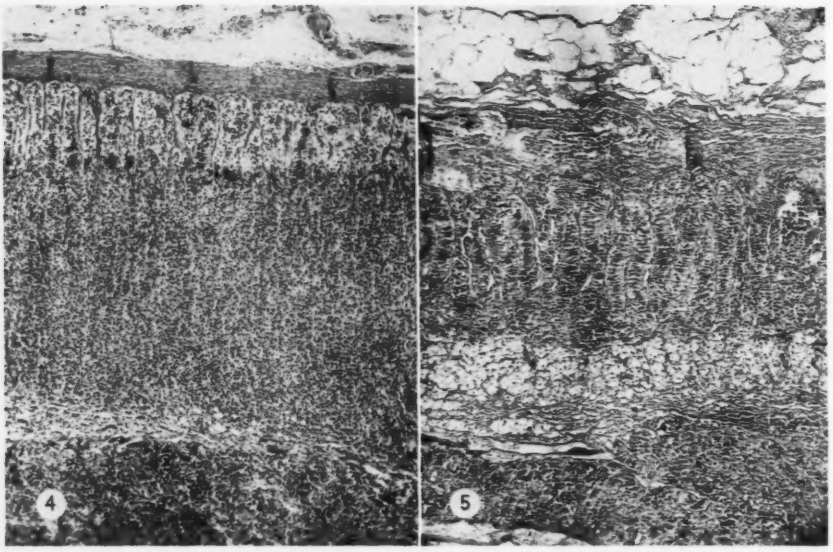
Comparison of products isolated. The infra-red spectrogram of the p,p′ perthane was that of a very pure sample of p,p′ perthane free of a significant quantity of the normal impurities (15). That of the synthetic product of o,p′ isomer obtained by Nichols and the mixture obtained by synthesis are practically identical (Fig. 2). The latter two

[3] The elemental analysis was done by Huffman Microanalytic Laboratories, Wheatridge, Colorado.

spectrograms show the characteristics expected from o,p′ isomer in the 735–860 Cm^{-1}, 1090–1225 Cm^{-1}, and 1600–2000 Cm^{-1} regions. The tracing of the o,p′ isomer which was isolated from the technical material was very similar to Nichols' product except for some additional bands suggestive of contamination by dehydrochlorinated p,p′ perthane and by 2,2 bis(p-acetylphenyl)-1,1-dichloroethane (15). Ultra-violet spectrograms of perthane isomers and dehydrochlorinated perthane were prepared with 2.5 $(10)^{-7}$ M solutions in pure ethanol using the Beckman DU spectrophotometer and taking readings every 5 microns from 210–300 mu (Fig. 3). The ultra-violet spectrogram of a sample of dehydrochlorinated perthane obtained from the Rohm and Haas Co., shows marked absorption in the ultra-violet region because of its conjugated system of double bonds (16). Similar absorption would be expected for the bis diacetyl diphenyl dichloroethane. From these spectrograms it was calculated on the basis of the absorption at 230 mu and 260 mu that the contaminating material may be present to an extent of 5–8% of the oil identified as consisting of relatively pure o,p′ perthane by paper chromatography.

Composition of technical perthane. The composition of the original batch of technical perthane was calculated from the weights of the products obtained after completing successive stages of the separation procedure. It was found to be: crystalline p,p′ isomer 69%, o,p′ isomer (oil) 8%, polar by-products 2%, unidentified material 21%. Perthane is said by the manufacturer to contain 87% p,p′ isomer and less than 2% 1-ethylphenyl-1,2,2-trichloroethane; the remainder being unidentified material (8). In comparison Cristol and Haller (17) isolated o,p′ DDD in 7–8% yield from technical DDD. Cueto and Brown (18) gave the composition of a batch of technical DDD to be p,p′ isomer 83%, o,p′ isomer 10%, the remainder being unidentified materials. Interfering side reactions probably account for some unrecovered material.

Administration to dogs. Technical perthane, recrystallized p,p′ isomer, and the residual oils were administered in hard gelatin capsules to mongrel dogs weighing 5–8 kgm. in a daily dosage of 50–200 gm./kgm as a 20% corn oil solution. Relatively pure o,p′ perthane isolated from the technical mixture and recrystallized p,p′ isomer were given as corn oil solutions by stomach tube to two litters of mongrel pups which had been weaned and pretreated with anti-canine hepatitis, and antidistemper serum. Slight contamination of the o,p′ isomer with dehydrochlorinated p,p′ perthane was not expected to affect interpretation of the results since the analogous product from DDD is without significant effect on the adrenal cortex (2). The administration of the perthane isomers and residual oils was continued for eight weeks or until the animals became moribund at which time they were sacrificed. As treatment progressed animals lost weight, showed muscle weakness and became ataxic. All dogs were autopsied and the adrenals removed, weighed, fixed in 10% formalin, and submitted for preparation of hematoxylin and eosin stained histological sections. All perthane fractions tested proved to have adrenocorticolytic properties (Figs. 4 and 5). Activity was judged by survival time and extent of histological damage. In Tables 1 and 2 four plus atrophy indicates that the cells of all three zones of the adrenal cortex exhibit extensive necrosis and loss of normal cellular pattern. Three plus atrophy indicates extensive damage confined to the zonae fasiculata and reticularis. Two plus atrophy



FIGS. 4 and 5. Histological sections of normal dog adrenal cortex (Fig. 4) and severely atrophied adrenal cortex (Fig. 5) after 6 weeks of treatment with p,p′ perthane (200 mg./kgm.). The normal adrenal cortex shows in order capsule, z. glomerulosa, z. fasiculata, the most extensive zone, and z. reticularis adjacent to the medulla. The atrophied adrenal cortex shows a thickened capsule, relatively intact z. glomerulosa, almost complete atrophy of z. fasiculata with vacuolization and fatty degeneration extending into z. reticularis.

indicates the presence of some necrotic changes in the inner two zones. The histological changes caused by technical perthane have been described in detail elsewhere (1). In Table 1 it will be noted that the residual oils were fed at a lower dosage than the technical material or the recrystallized p,p′ isomer. This was done with the thought that they would prove more potent. The data of Table 1 indicate that the residual oils, which consist principally of mixtures of the p,p′ and o,p′ isomers may be equally as potent as the recrystallized p,p′ isomer with respect to onset of severe toxic symptoms and degree of atrophy produced if fed at the same dosage. The residual oil, after removal of polar contaminants by chromatography on an alumina column, was as active as the crude residual oil. The data of Table 2 indicate that the relatively pure o,p′ perthane was also active, but may be somewhat less active than the p,p′ isomer. The two pups treated with o,p′ material were litter mates of similar weight and general condition as the pups treated with p,p′ material. They withstood a longer treatment period before terminal symptoms developed. Histologically, the adrenal damage appeared to be somewhat less extensive. Since the present study Nichols (14) has extended these observations with experiments on dogs

TABLE 1. EFFECT OF TECHNICAL PERTHANE AND FRACTIONS OBTAINED FROM IT ON THE ADRENALS OF MONGREL DOGS

Dog No.	Material administered	Dose (mg./kg.)	Wt. of dog (kg.)	Days of administration	Adrenal weight (gm./kg.)	Extent of adrenal atrophy
1†	technical perthane	200	5.8	17	—	+++
2†	technical perthane	200	7.5	8	0.074	+++
3	p,p′ perthane	200	6.5	42	0.063	++++
4†	p,p′ perthane	200	5.8	16	—	+++
5	p,p′ perthane	200	8.0	18	—	+++
6†	p,p′ perthane	200	7.0	12	0.098	+++
7†	p,p′ perthane	200	6.5	11	—	+++
8	residual oil I	200	7.0	10	0.076	+++
9	residual oil I	100	7.0	42	—	+++
10	residual oil I	100	7.5	44	0.114	+++
11	purified residual oil*	50	7.0	58	0.089	+++
12	corn oil only	—	9.0	58	0.131	0

* Purified by column chromatography on an alumina column petroleum ether fraction.
† Dogs nos. 1, 2, 4, 6, 7 were found dead in the cage. The other dogs were sacrificed when moribund.

using pure crystalline o,p′ isomer. On the basis of his findings he has also stated that the o,p′ isomer of perthane may be less active than the p,p′ isomer.

The results demonstrate that both the o,p′ and p,p′ isomers of perthane have adrenocorticolytic activity. Their combined effects are probably additive. In this respect perthane may differ from DDD in which it is claimed that o,p′ isomer is the potent fraction. The adrenocorticolytic activity of technical perthane is attributable to the content of these isomers. Because of the relative abundance of the p,p′ isomers as compared to the o,p′ isomers in the technical products, the p,p′ isomer of perthane may prove to be a more desirable material to use than the o,p′ isomer of DDD in experimental investigations for the production of adrenal cortical atrophy. This work further suggests that the structure activity relationships established for DDD may not be applicable to its analogs and indicates the need for further studies on the effect of structural variation of the DDD molecule on adrenocorticolytic activity.

TABLE 2. EFFECT OF O,P′ OR RECRYSTALLIZED P,P′ ISOMERS OF PERTHANE ON THE ADRENALS OF LITTER MATE WEANED PUPS

Pup	Sex	Litter	Wt. of pup (kg.)	Material administered	Dose (mg./kg.)	Days of administration	Extent of adrenal atrophy
1	M	A	3.1	p,p′ perthane	100	13	++
2	F	A	2.0	p,p′ perthane	100	12	+++
3	F	B	2.3	p,p′ perthane	100	11	+++
4	F	B	2.2*	p,p′ perthane	100	6	+++
5	M	A	2.0	o,p′ perthane	100	26	++
6	F	B	2.1†	o,p′ perthane	100	18	+++
7	M	A	2.0	control	—	26	0

* The feeding of the p,p′ perthane began when this pup was two weeks older than other pups used in the experiment.
† Pup No. 6 was found dead in the cage; others were sacrificed when moribund.

Addendum

After submitting the manuscript for publication, the author's attention was brought to some results of experiments in progress in the laboratory of Dr. John Nichols. He found that daily oral administration of pure p,p′ DDD, 500 mg./kg. in solution, to dogs, produced significant atrophy of the adrenal cortex. In contrast p,p′ DDD in a dosage of 200 mg./kg. was relatively ineffective. His work indicates that the effect of p,p′ DDD on the dog adrenal is dose dependent.

Acknowledgment

The author wishes to acknowledge the assistance of Dr. John Nichols, University of Kansas Medical Center, Department of Pathology, for his help in the histological examination of the slides and for his data on the o,p′ isomer. Dr. Daniel Turner, Jefferson Medical College, helped in interpreting the infra-red spectrograms, prepared by T. Sadtler and Son, Philadelphia, Pa.

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INHIBITION OF ERYTHROPOIESIS BY ESTROGENS

PETER P. DUKES AND EUGENE GOLDWASSER

Argonne Cancer Research Hospital,[1] and The Department of Biochemistry, The University of Chicago, Chicago, Illinois

ABSTRACT

Both natural and synthetic estrogens have been found to rapidly depress erythropoiesis in male rats. The doses employed and the time relationships obtained in these experiments suggest that estrogens are part of the regulatory mechanism governing red cell production.

THE administration of estrogens has been known to impair erythropoiesis in different animal species, when studied over rather long periods of time. Large doses of estrogens cause severe anemia in dogs (1, 2) and mice (3) but in the monkey the effect of similar doses is only slight (4). The regeneration of red cells was studied extensively in the rat (5, 6) and it was found that males recover from bleeding more rapidly than females. If bled female rats are given estradiol continuously they completely fail to restore their original red cell levels.

The conclusions drawn from these experiments, which utilized the usual indices of the peripheral blood (red cell count, hemoglobin concentration and hematocrit) in addition to studies of the morphology of the marrow was that the anemia resulting from estrogen administration is caused by the replacement of the marrow erythropoietic tissue by edema fluid or by bone.

By using a short method for determination of the rate of erythropoiesis (7, 8) we were able to study the mechanism of the inhibition of red cell formation by estrogens in more detail than had previously been possible. The results of the experiments reported here indicate that there is a rapid effect of estrogens on the marrow, which we suggest may compete with the effect of erythropoietin (9) in the maintenance of the steady state of erythropoiesis. A preliminary report of this work has already appeared (10).

MATERIALS AND METHODS

Male, Sprague-Dawley rats weighing 160 to 200 gm. (6 to 8 weeks old) were used in all experiments in groups of five or more. The animals received food (Purina rat chow) and water *ad libitum*. Test substances were injected subcutaneously, dissolved in peanut oil containing 3 mg. of benzyl alcohol per ml. However, erythropoietin and inorganic compounds were dissolved in saline. Erythropoiesis was measured by injecting 1 μc. of

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[1] Operated by The University of Chicago for the United States Atomic Energy Commission.

Fe^{59} Cl_3 into the tail vein of each rat, and 17 to 18 hours later removing a 1-ml. sample of blood by cardiac puncture. Radioiron was counted in a well-type scintillation counter. Results are expressed either as percent of the injected iron incorporated into the total red cell mass, or as percent depression of erythropoiesis with respect to the control group injected with only the vehicle.

Cell counts were done in a Coulter counter. Hemoglobin was determined colorimetrically as cyanmethemoglobin (11). Reticulocyte counts were made from wet mounts of whole blood stained with brilliant cresyl blue, and hematocrits were determined by the micro method.

We are indebted for the following gifts: Allylestrenol from Organon Inc., 4,4′-sec-butylidenediphenol, 4,4′-isopropylidenedicyclohexanol and p,p′-biphenol from the Dow Chemical Company. All other compounds were purchased from commercial sources.

RESULTS

After our observation that estradiol-17β in physiological amounts could depress the incorporation of Fe^{59} into red cells, we examined a number of other compounds, steroid and non-steroid for similar effect with the results seen in Table 1. The naturally occurring estrogens all were inhibitory as

TABLE 1. EFFECT OF ESTROGENS AND RELATED COMPOUNDS ON ERYTHROPOIESIS[1]

Compound	Dose/ rat (μg)	Control % Fe^{59} uptake	Experimental % Fe^{59} uptake	Standard error of difference[2]	% depression of Fe^{59} uptake
Estradiol-17β	20	46.1	34.0***	1.55	26
Estradiol-17β	20	42.5	29.3***	2.48	31
Estradiol-17β	20	43.7	32.5**	2.88	26
Estradiol Dipropionate	20	43.7	32.5*	4.26	26
Estradiol Dipropionate	20	30.6	23.5**	1.64	23
Ethinyl Estradiol	20	35.9	21.5***	2.13	40
Ethinyl Estradiol	20	43.7	21.5***	2.24	51
Estriol	20	46.1	34.9**	2.39	24
Estriol	20	43.7	33.3**	3.08	24
Estriol	20	35.9	32.0	2.17	11
Estriol	20	52.6	48.1	2.24	9
Estriol Glucuronidate	20	52.6	49.9	4.70	5
Estriol Glucuronidate	20	35.9	33.7*	0.95	6
Estriol Glucuronidate	20	43.7	39.8	2.48	9
Estrone	20	46.1	41.9	2.66	9
Estrone	20	42.5	39.2	1.71	8
Diethylstilbestrol	20	49.7	23.3***	5.59	53
Diethylstilbestrol	20	53.4	31.8***	3.01	40
Diethylstilbestrol	20	41.0	26.6**	3.30	35
Diethylstilbestrol	20	38.6	28.2***	1.26	27
Diethylstilbestrol	20	46.1	28.6**	4.02	38
Diethylstilbestrol	20	61.7	36.8***	2.35	40
Diethylstilbestrol	20	43.2	32.7**	2.69	24
Diethylstilbestrol	20	43.9	26.5***	1.67	40
Diethylstilbestrol	20	36.8	27.0*	3.05	27
Diethylstilbestrol	20	39.7	20.6***	2.39	48
Diethylstilbestrol	20	40.2	18.8***	1.94	53
Diethylstilbestrol	20	40.3	24.6***	1.91	39
Diethylstilbestrol	20	36.7	22.7***	1.41	38
Diethylstilbestrol	20	38.3	20.7***	3.50	46
Diethylstilbestrol	20	40.8	25.0***	2.07	39
Diethylstilbestrol	20	30.6	16.3***	1.90	47

TABLE 1 (*Continued*)

Compound	Dose/ rat (μg)	Control % Fe^{59} uptake	Experimental % Fe^{59} uptake	Standard error of difference[2]	% depression of Fe^{59} uptake
Diethylstilbestrol	20	38.3	20.5**	4.72	47
Dipropionate	20	40.8	20.6***	2.70	50
Hexestrol	20	61.7	41.8***	3.66	32
Dienestrol	20	61.7	49.9**	2.62	19
Dienestrol	20	38.3	22.9**	4.17	40
Dienestrol Diacetate	20	38.3	17.9***	4.04	53
Testosterone	20	61.7	53.2	4.00	14
Testosterone	20	42.5	43.0	2.10	− 1
Testosterone	20	39.7	43.3	2.92	− 9
Testosterone	20	38.3	31.7	4.89	17
Testosterone	20	36.6	40.7	1.25	−11
Testosterone Propionate	20	38.3	36.2	3.83	2
Progesterone	20	61.7	58.9	2.84	5
Progesterone	20	30.6	29.1	1.41	5
Allylestrenol	20	43.7	39.0	2.60	11
Allylestrenol	20	30.6	26.6*	1.30	13
Pregnenolone	20	38.3	36.4	3.90	5
Cortisol	20	46.1	45.7	1.45	1
Soybean Steroids (21% Stigmasterol)	20	42.5	43.7	2.36	− 3
4,4′-sec-Butylidenediphenol	20	38.3	39.7	4.68	− 4
4,4′-sec-Butylidenediphenol	100	40.8	33.2	4.59	19
4,4′-sec-Butylidenediphenol	100	30.6	29.3	1.18	4
4,4′-Isopropylidenediphenol	20	41.5	38.3	2.43	8
4,4′-Isopropylidenediphenol	20	46.8	40.8	1.56	13
4,4′-Isopropylidenediphenol	20	38.3	35.4	5.13	8
4,4′-Isopropylidenediphenol	20	36.6	38.1	1.39	− 4
4,4′-Isopropylidenediphenol	100	40.8	38.3	1.83	6
4,4′-Isopropylidenediphenol	100	30.6	28.6	1.31	7
4,4′-Isopropylidenedicyclohexanol	20	36.6	40.4	1.50	−10
	100	40.8	39.1	18.2	6
p,p′-Biphenol	20	38.3	35.7	3.50	7
p,p′-Biphenol	100	40.8	39.4	1.76	3
α-Naphtol	100	36.8	40.4	1.61	−10
β-Naphtol	100	36.8	40.7	2.22	−10
Sodium Thiocyanate[3]	87,000	43.9	37.6*	2.01	14
Sodium Thiocyanate[3]	87,000	41.5	34.0*	2.60	18
Sodium Thiocyanate[3]	87,000	46.8	40.0*	1.90	14

[1] Compounds were administered 72 hours before Fe^{59} injection.

[2] S.E. $(\bar{x}_1 - \bar{x}_2) = s\sqrt{\frac{1}{N_1} + \frac{1}{N_2}}$. N_1 and N_2 in all cases equals 5

* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.
No asterisk denotes $P > 0.05$.

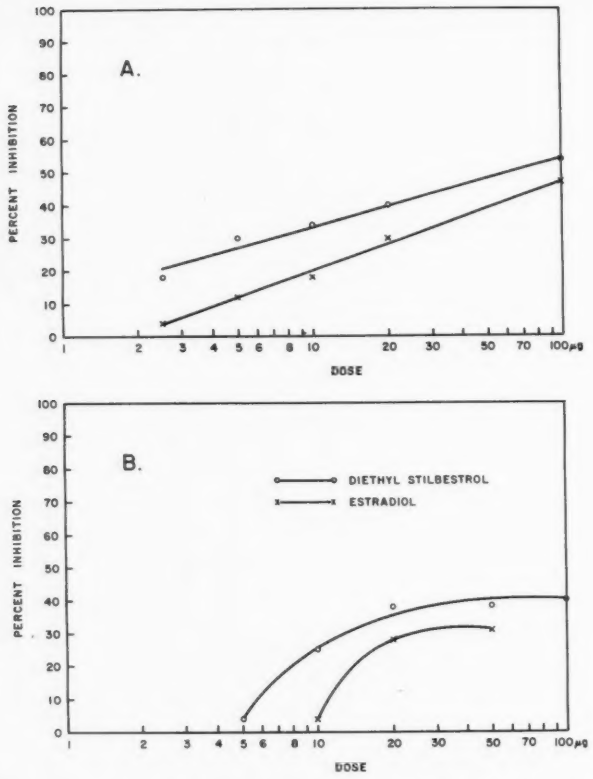

FIG. 1. Dose response relationship for estradiol and diethylstilbestrol effect on erythropoiesis. *A* compounds administered in 4 equal parts of the indicated dose on 4 consecutive days. *B* compounds administered in a single dose 3 days before Fe^{59} injection.

were the synthetic estrogenic compounds. Allylestrenol, which was found to be active is one exception in that it is not estrogenic but has progestational activity. Progesterone itself, however, is not inhibitory at the dose tested. Two compounds, 4,4′-sec-butylidenediphenol and 4,4′-isopropylidenediphenol show slight but definite activity at high doses. These data indicate that the structural requirement for erythropoiesis inhibition by this class of compounds involves one phenolic ring separated from an alicyclic or aromatic ring by a one- or more carbon atom bridge bearing short aliphatic side chains.

Conjugated forms of estrogens were, at least equally, as active as the parent compounds with the exception of estriol glucuronidate. Estrogens are much more active, on a molar basis, than NaCNS which was tested because of its known ability to direct the differentiation of amphibian embryonic tissue away from blood formation (12).

The dose-response relationships for diethylstilbestrol and estradiol are shown in Figure 1. In the upper part of the figure (A) the dose of estrogen was administered in 4 equal parts on consecutive days and the response is seen to be directly proportional to the logarithm of the dose. The lowest dose of estradiol with a detectable response is 2.5 μg per rat and, as will be seen below, the effective dose of estrogen was only 1.9 μg. In the lower part of Figure 1 both compounds were given in a single dose 72 hours before the radioiron. This injection schedule resulted in a lessened inhibition of erythropoiesis. Under both schedules diethylstilbestrol was a more active inhibitor of erythropoiesis than was estradiol.

The interval between administration of estrogen and of Fe^{59} has a pronounced effect on the magnitude of the inhibitory effect (Fig. 2). When 20

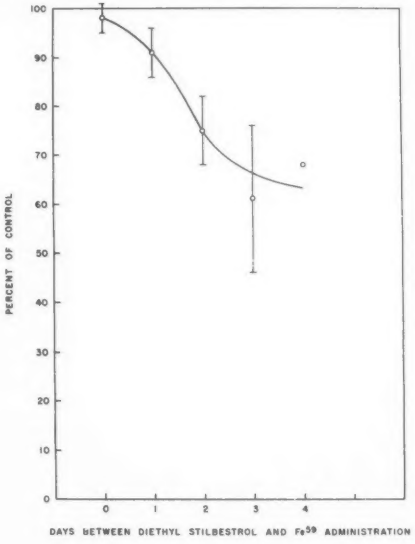

FIG. 2. Response to diethylstilbestrol as a function of time between administration of estrogen and injection of radioiron. At each point where applicable, 1 standard deviation from the mean is indicated.

μg. of diethylstilbestrol are injected 30 to 60 minutes before the assay of erythropoiesis is started, the iron incorporation remains at the control level. This finding indicates that the last dose used in the experiment described in Figure 1A was without effect and that only 75% of the injected estrogen was needed to produce the observed inhibition. Maximal inhibition of erythropoiesis occurs when the interval between a single injection of diethylstilbestrol and the injection of radioiron is three days. These findings suggest that diethylstilbestrol acts to inhibit an event concerned with the earliest phase of red cell development in the marrow. The stimulation of red cell formation by erythropoietin has been shown by several investigators to similarly involve an early step in erythropoiesis (13, 14, 15, 16).

The stimulation of red cell formation by erythropoietin can be reversed

TABLE 2. EFFECT OF DIETHYLSTILBESTROL ON RESPONSE TO ERYTHROPOIETIN[1]

	% Fe^{59} uptake	P
Control	37 ± 3	
5 Units Sheep Erythropoietin	37 ± 9	—
10 Units Sheep Erythropoietin	42 ± 2	0.02 < 0.01
5 Units Sheep Erythropoietin plus 20 μg Diethylstilbestrol	30 ± 4	0.01 < 0.001
10 Units Sheep Erythropoietin plus 20 μg Diethylstilbestrol	30 ± 4	0.01 < 0.001
20 μg Diethylstilbestrol	23 ± 2	< 0.001

[1] Diethylstilbestrol was injected on the third day and erythropoietin in 2 equal doses on the second and first day before Fe^{59} administration.
Results expressed as mean ± standard deviation.

by administration of estrogens, and the inhibitory action of estrogens can be partly overcome by the amount of erythropoietin used (Table 2). This relationship is noted whether partially purified sheep erythropoietin (9) is administered exogenously or whether the hormone is formed endogenously as a result of cobaltous ion administration (17). It should be mentioned that the doses of erythropoietin (5 and 10 units (18)) used in these experiments are quite large. In the standard, starved rat assay (8) 5 units of erythropoietin would elevate iron incorporation from the control value of 4 to 5% to about 18% of the injected Fe^{59}. Non-starved rats as used in the present study are much less responsive to exogenous erythropoietin and it takes 10 units to exceed the control value of iron uptake significantly. On the other hand, 10 units of erythropoietin can only partially reverse the depression caused by 20 μg of diethylstilbestrol.

The effect of increasing amounts of cobalt, with a constant amount of diethylstilbestrol compared with groups of rats receiving no diethylstilbestrol is seen in Table 3. While the response to the cobalt is slight in the animals not treated with diethylstilbestrol, the rats treated with diethylstilbestrol show a more pronounced response to the same level of cobalt. If

TABLE 3. ANTAGONISM BETWEEN $CoCl_2$ AND DIETHYLSTILBESTROL[1]

μMoles Co^{++}	% Fe^{59} uptake after administration of	
	No diethylstilbestrol	20 μg diethylstilbestrol
0	41 ± 3	27 ± 3**
5	45 ± 4	34 ± 5**
10	46 ± 2	36 ± 6**
15	47 ± 4	40 ± 4*
20	49 ± 4	39 ± 4**

[1] Diethylstilbestrol was injected on the third day and $CoCl_2$ in two equal doses on the second and first day before Fe^{59} administration.
* $P < 0.05$.
** $P < 0.01$.
Results expressed as mean ± standard deviation.

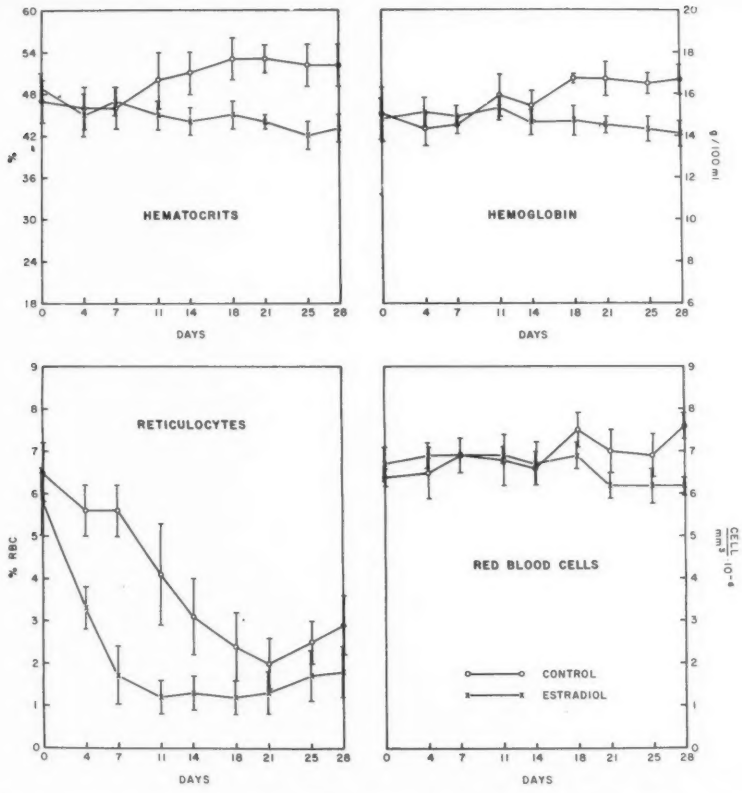

FIG. 3. Effect of continued administration of estradiol on red cell indices. Groups of 12 rats were used. One standard deviation from the mean is indicated. On day 28 the differences between untreated and treated groups were: hematocrits, −9 (% Het.); Hemoglobin, −2.6 (g/100 ml); Red blood cells, −1.4 (cell/mm³ 10^{-6}); P in all cases <0.01. Difference in reticulocyte count on day 7 (the day of greatest difference) was −3.9 (%R.B.C.) P <0.001.

conditions could be found to magnify these differences another modification of the Fe^{59} assay method for erythropoietin might be developed which could eliminate the use of hypophysectomized (19) or starved (8) animals and therefore have the advantage of using a sturdier assay animal.

Thiocyanate has a similar effect to diethylstilbestrol in reversing the action of cobaltous ion on Fe^{59} incorporation. For instance, 15 μMoles of $CoCl_2$ injected in two parts, 1 and 2 days before Fe^{59} injection, increased Fe^{59} uptake from 40 ± 3 to $47 \pm 3\%$ (one standard deviation indicated) ($P < 0.01$). If in addition to this schedule of $CoCl_2$ injection, 87 mg. NaCNS, were administered distributed over the 3 days previous to Fe^{59} injection, the incorporation changed from $47 \pm 3\%$ to $37 \pm 4\%$ ($P < 0.01$).

In a longer study we have seen that estradiol can appreciably effect a lowering of the peripheral blood indices of red cell production. Each treated rat was given a daily injection of 10 μg of estradiol in 1 ml. of peanut oil while control rats were given peanut oil alone. The results of this experiment (Fig. 3) indicate a rapid interference with erythropoiesis by estradiol as seen by the sharp fall in reticulocytes while the red cell count, hemoglobin and hematocrit levels fell more slowly. The control group also shows a decrease in the number of reticulocytes; this can perhaps be explained (aside from a normal decrease with age) by the recently reported finding (20) that at least in the mouse peanut oil itself has a slight estrogen-like activity. At the end of the 28 days the Fe^{59} incorporation in the treated groups was 21 ± 6 while that for the control group was 34 ± 8 (mean $\pm$standard deviation indicated; $P < 0.02$). The estradiol group had an average body weight of 263 gm. while the control group averaged 318 gm. This slower increase in body weight exactly balanced the fall in red cell mass, measured by dilution of Fe^{59}-labeled red cells and the red cell volume as % of body weight remained the same for both groups, 4.8–4.9%. The absolute red cell mass of the estradiol treated group was about 12 gm. in contrast to the control group with 16 gm.

DISCUSSION

The normal rate of erythropoiesis is maintained by a constant discharge of erythropoietin from the kidney (9) and possible other sources (21, 22) into the circulation. Superimposed upon this control mechanism there is, we suggest, a negative control by way of estrogenic substances acting on the marrow. The lower red cell levels of females would support this contention. In order to keep the system which produces erythropoietin active and in a state capable of responding to hypoxia by producing increased quantities of the stimulatory substance and yet not produce an excess of red cells the organism would require a method of neutralizing the effect of erythropoietin. Estrogens may serve this purpose acting to repress the differentiation of the multipotent stem cells toward the red cell line. Estradiol and thiocyanate have been shown by Runnström and Kriszal (23) to suppress differentiation of sea urchin egg endodermal tissue and we have seen that they also depress red cell formation. Similarly, Ken-ichi Ôgi (12) has shown that thiocyanate treated medio-ventral marginal zone explants of Triturus embryo showed a definite reduction in formation of blood islands after nine days of cultivation.

The decreased weight gain observed in the estradiol treated group of the 28 day experiment points to the fact that not only the erythropoietic system is adversely affected by estrogens. Attention is called to experiments by Priest and co-workers (24) showing that estradiol reduces the incorporation of radioactive sulfate into cartilage and aortas of rats.

While the problem is still very far from being settled we feel that the

study of red cell formation offers the possibility of new insights into the molecular mechanism of differentiation.

Acknowledgment

We wish to thank Dr. Leon O. Jacobson for advice and Dr. Paul Meier for advice concerning the statistical treatment used. The technical assistance of Miss Sandra Hamilton is gratefully noted.

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THE MECHANISM OF PROTEIN IODINATION DURING THE METABOLISM OF THYROID HORMONES BY PERIPHERAL TISSUES[1]

VALERIE ANNE GALTON[2] AND SIDNEY H. INGBAR[3]

Thorndike Memorial Laboratory, Second and Fourth (Harvard) Medical Services, Boston City Hospital, and the Department of Medicine, Harvard Medical School, Boston, Massachusetts

ABSTRACT

During the metabolism of I^{131}-labeled thyroxine and its analogues by tissue preparations, two major products are formed: inorganic iodide and a material which remains at the point of application during paper chromatography. The nature of this "origin material" and the mechanism of its formation have been investigated. The I^{131} in origin material was TCA-precipitable, non-dialyzable and was not extractable with lipid solvents, and was therefore thought to be an iodinated protein. Except in the presence of serotonin, the formation of origin material during the metabolism of thyroid hormones was invariably associated with the appearance of inorganic iodide. However, origin material was not found when inorganic iodide was the initial substrate. Incubation in complete darkness had no consistent effect on the generation of either iodide or origin material from thyroxine. Furthermore, anaerobiosis and goitrogens failed to prevent the formation of origin material from labeled thyroxine. It was thus concluded that origin material is not formed by the re-oxidation of iodide liberated during hormonal deiodination. An alternative mechanism is proposed and its implications with regard to hormonal action are discussed.

IN RECENT years, much information has been obtained concerning the metabolism of the thyroid hormones by peripheral tissues. The degradation of thyroxine (T4), 3,5,3′-triiodothyronine (T3) and their analogues by peripheral tissue preparations has been widely studied *in vitro*, and it has been established that many mammalian and avian tissues possess systems for deiodinating or deaminating these compounds. These findings have recently been reviewed (1).

During these studies, other workers have noted that when I^{131}-labeled T4 or its derivatives are incubated with tissue preparations, labeled material is often formed which remains at the point of application during chro-

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[2] Milton Research Fellow, Harvard University.

[3] Investigator, Howard Hughes Medical Institute.

matographic analysis (1–5). In preparations of tissues from the rat, as much as 20% of the total radioactivity is present in this fraction (5).

Several authors have thought that the origin material is an iodinated protein, largely because of its insolubility in organic solvents (5, 6). However, the mechanism by which protein may be iodinated during the deiodination of T4 has received relatively little attention. Two possible mechanisms have been suggested (7). First, proteins might be directly iodinated by an immediate product of T4 deiodination, or, secondly, iodide liberated in the course of deiodination might undergo reoxidation and then iodinate protein(s) in the tissue preparation.

During studies in this laboratory of the metabolism of thyroid hormones by mammalian and amphibian tissues, it was observed that the formation of origin material was almost always associated with deiodination of the hormones. Formation of inorganic iodide and origin material increased with time and the latter comprised as much as 30% of the total I^{131} present. However, certain observations made it seem unlikely that origin material indeed results from the re-oxidation of iodide liberated during hormonal degradation. First, previous workers had shown that tissues later demonstrated to form origin material from T4 were unable to produce significant quantities of protein-bound iodine when inorganic iodide was the substrate (8). Second, during studies of the effects of tryptophane derivatives on the metabolism of T4 *in vitro*, it was observed that serotonin could completely inhibit the appearance of inorganic iodide while increasing the formation of origin material (7).

Two types of experiments were performed in order to assess more critically the mechanism by which origin material is formed. First, the influence on origin material formation from T4 of several conditions known to inhibit the oxidation of iodide was determined. Second, comparisons were made in the same tissue preparation of the amount of origin material formed when inorganic iodide or thyroxine was employed as substrate.

MATERIALS AND METHODS

Studies were performed in specimens obtained from rats, mice, tadpoles and frogs. Tissues were quickly removed and homogenized (1:10, w/v) in ice-cold Krebs-Ringer phosphate buffer.[4] Boiled homogenates were re-homogenized before use.

Immediately upon arrival from a commercial source,[5] radioactive compounds, other than sodium iodide, were diluted with 1% human serum albumin to give a final con-

[4] Krebs-Ringer-phosphate buffer, pH 7.4:

0.154 M NaCl	100 parts
0.154 M KCl	4 parts
0.11 M $CaCl_2$	1 part
0.154 M $MgSO_4$	1 part
0.1 M sodium phosphate buffer pH 7.4	10.6 parts

[5] Obtained in solution in 50% propylene glycol from Abbott Laboratories, Oak Ridge, Tennessee. According to chromatographic analysis, 85–95% thyroxine, with the major contaminant inorganic iodide.

centration of 160 μc./ml., a procedure found to retard chemical decomposition. Subsequently, small aliquots of these solutions were added to homogenates (25 μl./ml.) to yield final concentrations of $1–4\times10^{-7}$ M. I^{131}-labeled sodium iodide, as obtained from the same commercial source, was added to homogenates together with sufficient stable iodide to yield a final concentration of 1×10^{-7} M. In some experiments, human serum albumin or stable T_4 was added to flasks containing inorganic I^{131}. In the latter instances, the hormone was dissolved in 50% propylene glycol and added to a concentration of $1–4\times10^{-7}$ M.

When employed, tapazole, propylthiouracil or serotonin were added to yield a concentration of 10^{-3} M. In all instances, the pH of the added solution was adjusted to 7.4 prior to addition.

Except when otherwise noted, specimens were incubated at 37° C in a metabolic shaker under room air for up to 4 hours. Following incubation, human plasma (0.5 ml./1.0 ml. homogenate) was added to each flask. This served to stop further reaction and also to reverse any spontaneous chemical alteration of the substrate which may have occurred (9). Aliquots of the mixtures were applied directly to strips of Whatman No. 1 filter paper for chromatographic analysis. The solvent systems used were butanol-dioxane-2N NH_4OH (4:1:5), and butanol-2N acetic acid (1:1). Following chromatography, strips were scanned in a strip-scanner and the areas under the several radioactive peaks were identified and quantified by methods previously described (10). In some experiments, the percent of organically-bound I^{131} as determined chromatographically (T_4+origin material) was compared with that estimated by trichloroacetic acid (TCA) precipitation.

Several experiments were performed in a completely blackened room. Here, T_4 was added to tissue homogenates in the dark and incubations were performed for one hour, after which serum was added and the resulting mixture was applied to chromatography paper. Light was admitted to the room only after the material applied to the paper was completely dry. Results were compared with those obtained in the same homogenate during comparable, concomitant incubation under a bright fluorescent light.

RESULTS

Except in the case of tissues incubated with serotonin, the formation of origin material was invariably associated with the appearance of iodide (Fig. 1). Thus, homogenates of tadpole, rat and mouse liver all generated inorganic I^{131} from labeled T4, and all produced origin material.[6] In boiled homogenates of tadpole and rat liver, hormonal degradation was inhibited and no origin material was formed. The ability of some boiled tissue preparations to degrade T4 has been noted by others (11–14). Boiled homogenates of mouse liver continued to form both inorganic iodide and origin material. On the other hand, in tissues which did not metabolize T4, such as frog liver (15) and sheep thyroid (16), no origin material was formed.

Considerable amounts of origin material were also formed when homogenates of tadpole liver were incubated with T3, triiodothyroacetic acid (TA3) and tetraiodothyroacetic acid (TA4) (Fig. 2). In this preparation, origin material was also formed from diiodotyrosine (DIT) amd MIT.

[6] All experiments described were performed on at least 4 separate occasions. Results which formed the basis for conclusions concerning the formation of origin material were highly reproducible.

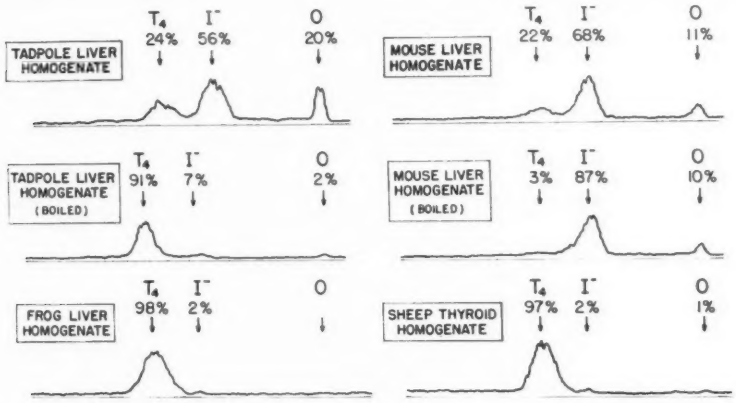

FIG. 1. Formation of origin material during the incubation of various tissues with I^{131}-labeled thyroxine. Solvent system: butanol-dioxane-2N NH_4OH (4:1:5). O—origin, I^-—inorganic iodide, T_4—thyroxine.

However, preliminary experiments suggest that the mouse liver homogenate, though capable of generating inorganic iodide, does not form origin material from MIT and DIT.

Figure 3 depicts typical results obtained when a homogenate of tadpole liver was incubated with either labeled sodium iodide (1×10^{-7} M) or labeled T4 (2×10^{-7} M). Origin material was not formed when iodide was the substrate. On the basis of the quantity of iodide found in the boiled

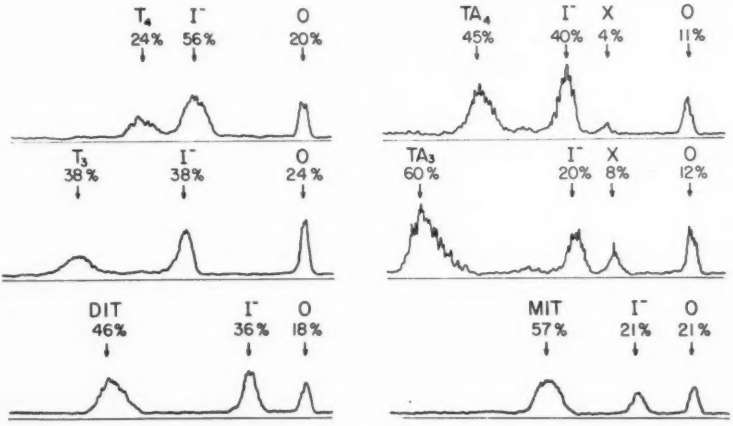

FIG. 2. Formation of origin material during the deiodination of iodinated compounds by tadpole liver homogenate. Solvent systems: butanol-dioxane-2N NH_4OH (4:1:5), and butanol-2N acetic acid (1:1). O—origin, I^-—inorganic iodide, T_4—thyroxine, T_3—3,5,3′ triiodothyronine, TA_4—tetraiodothyroacetic acid, TA_3—3,5,3′ triiodothyroacetic acid, MIT—monoiodotyrosine, DIT—diiodotyrosine.

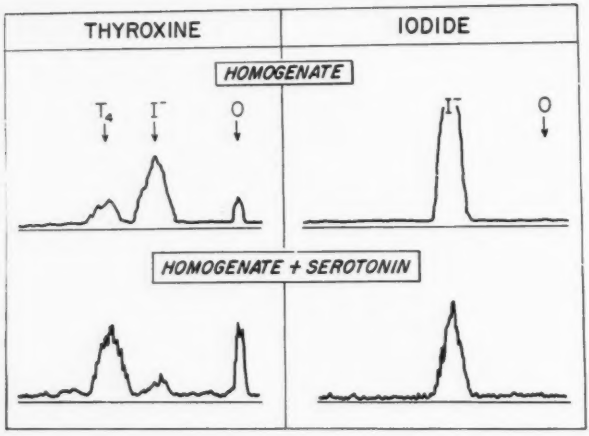

FIG. 3. The effect of serotonin on the metabolism of I^{131}-labeled iodide and thyroxine by tadpole liver homogenate. Solvent system: butanol-dioxane-2N NH_4OH (4:1:5). O—origin, I^-—inorganic iodide, T_4—thyroxine. Iodide seen in the serotonin-T_4 specimen comparable to that found in boiled specimens.

control, serotonin (10^{-3} M) completely prevented the generation of inorganic iodide from T4 while increasing the formation of origin material. However, it did not induce the formation of origin material when the homogenate was incubated with inorganic iodide. In other experiments, addition of stable T4 (1×10^{-7} M) did not stimulate the formation of origin material from inorganic iodide. Thus, the formation of origin material could not have been due solely to the higher concentration of thyroxine in those flasks in which I^{131}-labeled T4 was the substrate.

In homogenates of tadpole liver, generation of inorganic iodide was not inhibited by anoxia, proplythiouracil or tapazole; in some experiments it appeared to be stimulated. These factors, which inhibit iodinations in the thyroid gland, did not prevent the formation of origin material from T4 (Fig. 4).[7]

Neither deiodination nor the formation of origin material was significantly or consistently influenced by incubation of tissue preparations in complete darkness, as compared to bright light (Table 1).

Origin material was not dialyzable and was not extracted from whole homogenates by lipid solvents (chloroform, chloroform-methanol, ether). A comparison of chromatographic results with those obtained by TCA-precipitation indicated that the origin material was TCA-precipitable. Attempts to hydrolyze the origin material failed. Incubation with pancre-

[7] In some experiments, anoxia inhibited hormonal degradation by tadpole liver. However, this effect was not consistent. In the experiment shown, anoxia completely inhibited the generation of iodide and origin material in comparably and concomitantly incubated homogenate of mouse liver.

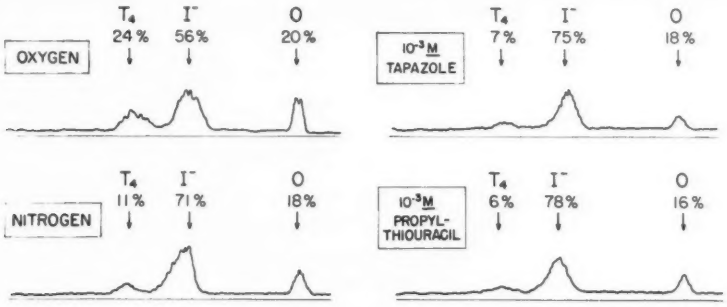

FIG. 4. Formation of origin material during incubation of tadpole liver homogenate with I^{131}-labeled thyroxine. The effect of anaerobiosis and antithyroid agents. Solvent system: butanol-dioxane-2N NH_4OH (4:1:5). O—origin, I^-—inorganic iodide, T_4—thyroxine.

atin, purified trypsin, or pepsin had no effect, even when the homogenate was first treated with ribonuclease, desoxyribonuclease, desoxycholate or sonication. Treatment with acid or alkali either had no effect or resulted in the appearance of inorganic iodide.

DISCUSSION

The precise nature of the iodinated material which is formed during the metabolic degradation of T4 and which remains at the origin of chromatograms is unknown. It has been suggested that the origin material is comprised of iodinated protein (5, 6) and the findings in this study support this view. However, the present attempts to hydrolyze the origin material have failed.

In the present experiments, origin material was formed only in tissue

TABLE 1. THE EFFECTS OF INCUBATION IN LIGHT AND DARKNESS ON THE METABOLISM OF I^{131} LABELED THYROXINE BY HOMOGENATES OF TADPOLE LIVER

Experiment No.		Radioiodinated compounds in homogenate after incubation* (% total I^{131})		
		T4	I^-	Origin material
1	Light	46	63	21
	Dark	53	38	9
2	Light	46	35	19
	Dark	45	32	23
3	Light	48	36	16
	Dark	48	32	20
4	Light	60	25	15
	Dark	60	24	16

* Values shown corrected for iodide and origin material found in comparably-treated specimens boiled prior to incubation.

systems capable of producing inorganic iodide from T4 and its derivatives. However, tissues capable of forming origin material during incubation with T4 were unable to do so when inorganic iodide was the initial substrate. Furthermore, serotonin enhanced the formation of origin material from T4, although it completely inhibited the generation of iodide. This effect was not due to increased conversion of iodide to origin material, since the same tissue preparation failed to form origin material from added iodide, even in the presence of serotonin. Finally, anaerobiosis and the addition of goitrogens, which prevent organification of iodine in the thyroid, failed to prevent the formation of origin material from T4 in tissues capable of degrading the hormone under these circumstances. For these reasons, it appears unlikely that origin material is formed from the inorganic iodide liberated during the deiodination of T4.

The viewpoint of other workers with regard to the mechanism of formation of origin material has not been clear. In 1959, Tata reported that the deiodinating activity of a rabbit skeletal muscle preparation was increased by the addition of either flavins or ferrous ions (6). However, while the formation of origin material was enhanced by the addition of flavin, no increase occurred in the origin material formed in the presence of iron, although deiodination was markedly stimulated. Tata remarked that this effect resembled the increased protein-bound I^{131} formed in thyroid homogenates with inorganic I^{131} in the presence of flavin mononucleotide (FMN). From this comment, it was not clear whether he felt that inorganic I^{131} is also the substrate for protein iodinations in the T4-deiodinating system, as in the thyroid homogenate. However, it appeared possible that this was indeed the case, and that the flavin-induced increase in origin material, described by Tata, was the result of a non-enzymic, light-dependent oxidation of iodide by flavins, similar to that described by Soodak and Maloof (17). Mixtures of inorganic iodide and flavin are capable of iodinating added protein when exposed to light (18). The possibility that a similar mechanism may have been operative in Tata's experiments can not be excluded since the experiments reported were carried out only in the presence of light and iodide was not employed as substrate.

This mechanism, however, could not account for the results obtained in the present experiments, in which no flavins were added, since no origin material was formed from inorganic iodide, even when homogenates were incubated in bright light. Furthermore, complete darkness failed to influence the protein iodinations which took place concurrent with the deiodination of T4.

Therefore, if, as the present data indicate, origin material need not arise from inorganic iodide, then the deiodination of T4 must proceed in one of two ways. First, deiodination may yield two products: inorganic iodide and a compound which leads to the formation of origin material. Alterna-

tively, deiodination may yield a single product which then gives rise to both origin material and iodide. If the origin material is indeed iodinated protein, then, in peripheral tissues, as in both the thyroid gland and non-living systems, the iodinating agent must be an oxidized form of the halogen. It may be concluded, therefore, that a relatively oxidized form of iodine is at least one of the products of hormonal deiodination.

In vitro, oxidation of iodide is required for aromatic iodinations, and physico-chemical principles indicate that a similar oxidation occurs in the thyroid gland during the biogenesis of T4. The nature of the actual iodinating agent is unknown, but it is thought to be either molecular iodine, or more likely, the hypoiodous acidium ion, IOH_2^+, or the iodinium ion, I^+ (19). It would therefore be anticipated, by the principle of "microscopic reversibility" (19), that the same iodinating agent would be the initial product of hormonal deiodination. This would have the ability to iodinate tissue proteins, thereby forming origin material, or to undergo reduction, forming inorganic iodide.

The formation of origin material, and presumably the generation of the hypothetical iodinating agent, is not an artifact of *in vitro* deiodination, since origin material has been found in a variety of tissues from animals given I^{131}-labeled T4 or T3 *in vivo* (20). Recent studies in this laboratory have led to the hypothesis that deiodination of the thyroid hormones is requisite to their biological action (7, 15). If this is the case, then it may be that the iodinating agent is the active principle of the thyroid hormone.[8] On the other hand, hormonal action may result from those processes which are associated with the reduction of this agent to iodide. While these considerations are speculative, their heuristic value is apparent.

Addendum

Since this manuscript was submitted, Tata has reported that, in the presence of FMN, indifferent proteins, such as lysozyme and egg albumin, are iodinated during the deiodination of T4 by a preparation of thyroxine dehalogenase (22). These iodinated proteins yielded monoiodotyrosine on tryptic hydrolysis. On the basis of unpublished data, Tata concluded that inorganic iodide was not the substrate for protein iodinations in the deiodinating system, a conclusion with which the present findings are in agreement.

Acknowledgments

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[8] The recent demonstration that large doses of inorganic iodide promote growth in thyroidectomized rats (21) also raises the possibility that some form of iodine *per se* is responsible for the action of thyroid hormone.

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ISOLATION OF 18-HYDROXYDEOXYCORTICOSTERONE FROM RAT ADRENALS[1]

FERNAND G. PÉRON
With the Technical Assistance of WILLIAM F. ROBIDOUX
The Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts

ABSTRACT

Substance X_2, a steroid elaborated by the rat adrenal gland which reacts with the sulfuric acid phenylhydrazine (PS) reagent yielding a chromogen absorbing light at 400–410 mμ. has been identified as 20,21-dihydroxy-18,20-epoxy-4-pregnene-3-one (18-hydroxydeoxycorticosterone). No chemical reason has been found why the cyclohemiketal grouping should facilitate the formation of an intermediate (for e.g. glyoxal) which would react with the PS reagent. Since 18-hydroxydeoxycorticosterone was derived solely from endogenous precursor(s) and because its biosynthesis can be increased by adrenocorticotropin, adenosine-3′,5′-monophosphate and reduced triphosphopyridine nucleotide, it is a natural adrenocorticosteroid and more than likely plays an important function in the physiology of the rat.

THE isolation and identification of some adrenocorticosteroids released by rat adrenal tissue incubated *in vitro* has been reported (1). Aldosterone and corticosterone were unequivocally identified by using paper chromatographic and some of the classical techniques. Another compound of unknown structure designated as X_2, was isolated in crystalline form. It reacted with the phenylhydrazine sulfuric acid reagent of Silber and Porter (PS) (2) giving rise to a chromogen absorbing light maximally at 400–410 mμ. Since it did not have the same structure as any of the adrenal steroids known to react with the PS reagent as indicated above, an elucidation of its structure was sought. Also, knowledge that there is a greatly increased biosynthesis of this substance by rat adrenal glands incubated *in vitro* in the presence of adrenocorticotropin (ACTH) (3) leads one to the point of view expressed previously (1) that it may be important *in vivo* in the overall physiology of the rat. Therefore, in the light of these facts, attempts at identifying this substance appeared justified.

During this study it was kept in mind that the substance could possibly be a steriod glyoxal because Lewbart and Mattox (4) have shown recently that the latter readily react with the PS reagent giving rise to chromogenic compounds which absorb light at or around 410 mμ.

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For reasons discussed previously and due to the absence of a 20-keto carbonyl absorption band in the infrared spectrum of X_2 (1) 18-hydroxyprogesterone in the cyclic 18–20 cyclohemiketal form (5, 6) was also considered as a possibility. However, the much lower polarity of this substance compared to X_2 in various paper chromatographic solvent systems eliminated 18-hydroxyprogesterone as being the substance whose structure was sought.

Identification of X_2 was finally made possible by isolating and identifying its periodate oxidation product by paper chromatographic and infrared spectroscopic techniques as well as comparing certain physical characteristics of X_2 with authentic 18-hydroxydeoxycorticosterone. This resulted in showing conclusively that X_2 is 20,21-dihydroxy-18,20-epoxy-4-pregnene-3-one (18-hydroxydeoxycorticosterone).

MATERIALS AND METHODS

Substance X_2 which was used in this study was that isolated previously (1) with a melting point of 168–171°. Authentic 20-hydroxy-18,20-epoxy-4-pregnene-3-one (18-hydroxyprogesterone) was a generous gift from Dr. L. Lábler of the Czechoslovak Academy of Science, Praha, Czechoslovakia (5) while authentic 18-hydroxydeoxycorticosterone was obtained from Dr. Raphael Pappo of G. D. Searle & Co., Chicago, Illinois (6).

A small amount of X_2 (450 μg.) dissolved in 1.0 ml. of methanol was oxidized by adding 0.65 ml. of a solution of 500 mg. of sodium metaperiodate in 50 ml. of water containing 2 drops of 2N sulfuric acid. After standing at room temperature in the absence of light for 18 hours the reaction mixture was extracted with dichloromethane. After washing the latter with 10% sodium hydrosulfite in 0.5N sodium hydroxide and then with water to neutrality, the neutral extract was brought to dryness under reduced pressure. The residue was used as is described in the results section below. Chromic acid oxidation of 18-hydroxyprogesterone was carried out by dissolving 1.0 mg. of this substance in 2.0 ml. of a solution of 150.0 mg. chromium trioxide dissolved in 50.0 ml. of glacial acetic acid. After standing at room temperature for 15 minutes the reaction mixture was extracted with dichloromethane and the latter washed with cold 1N sodium hydroxide followed by water to neutrality. The neutral extract was worked up in the same manner as for the neutral extract of X^2 oxidized above.

The paper chromatographic methods employed for the separation of the reaction products of oxidation were the Zaffaroni- (7) or Bush-type (8) systems. These were run at 28° in a constant temperature cabinet. In all cases the developed chromatograms were scanned with ultraviolet light and sprayed with 10% aqueous sodium hydroxide (NaOH reagent) (8) in order to visualize the position occupied by the compounds on the chromatograms.

RESULTS

The neutral residue obtained from the oxidation of 450 μg. of X_2 (oxidized X_2) was chromatographed in the ligroin propylene-glycol system for 64 hours (100% impregnation with propylene-glycol). One distinct zone absorbing ultraviolet light was visualized which had a relative mobility of 0.49 when deoxycorticosterone was equal to 1. A sample strip cut from the main limb of the chromatogram showed this zone to be strongly fluorescent

after spraying with the NaOH reagent. After rechromatography of the eluate in the same system in order to ensure its purity, aliquots of the ultraviolet light absorbing material were run in the Bush B_3 and the cyclohexane formamide-benzene (20% impregnation with formamide) systems. In these systems oxidized X_2 had a relative mobility of 0.90 and 0.93 respectively when the mobility of deoxycorticosterone was equal to 1. In each case only one spot absorbing ultraviolet light could be visualized on the paper chromatograms which coincided exactly with the spot giving a good soda fluorescence after spraying with the NaOH reagent. Oxidized X_2 readily crystallized from methanol in the form of rosettes or tufts and had a melting point of 221–226° (uncorrected). In contrast to the original X_2, this product no longer gave a positive PS reaction and afforded infrared spectrum B (KBr pellet) shown in Figure 1.

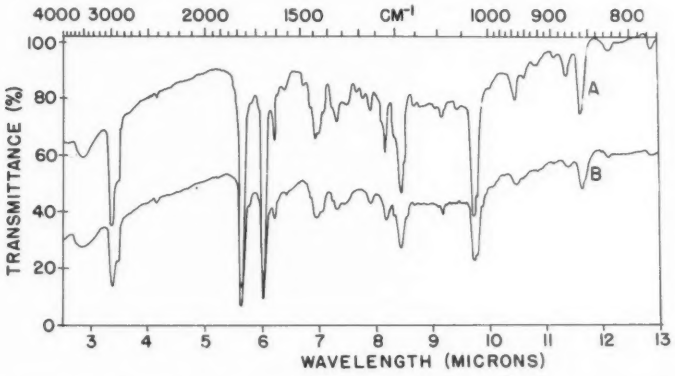

FIG. 1. Tracing of KBr infrared spectra on Perkin-Elmer infracord spectrophotometer. A, lactone from 18-hydroxyprogesterone. B, lactone from X_2.

The chromic acid oxidation product of 1.0 mg. of 18-hydroxyprogesterone (see Methods) was chromatographed in the ligroin propylene-glycol system as in the above for oxidized X_2 and isolated. The mobilities of this product when run singly or admixed with oxidized X_2 in the different chromatographic systems mentioned were identical with those given for oxidized X_2. Its melting point as well as its infrared spectrum were identical to that obtained for oxidized X_2 (cf. Fig. 1, Spectrum A).

Further proof establishing the structure of X_2 was obtained after receipt of the authentic 18-hydroxydeoxycorticosterone. The infrared spectrum of this compound (KBr pellet) was almost superimposable on that reported for X_2 (1) except that the 1745 cm.$^{-1}$ band was much weaker while no band was observed at 1720 cm.$^{-1}$ (1). Both authentic 18-hydroxydeoxycorticosterone and X_2 had identical mobilities in the toluene propylene-glycol, Bush B_3 and B_8 chromatographic systems. No separation of the compounds

was obtained when they were run in these systems as admixtures. Periodate oxidation of 18-hydroxydeoxycorticosterone also yielded a product identical in mobility and having the same infrared spectrum as oxidized X_2.

Finally, the products obtained from the acetylation of X_2 and authentic 18-hydroxydeoxycorticosterone in pyridine and acetic anhydride for 16 hours at room temperature, had identical mobilities when run as single substances or as admixtures in the Bush B_2, B_3 and the cyclohexane formamide-benzene systems (40% impregnation with formamide) (0.99, 1.05 and 1.25 respectively, when the mobility of corticosterone-21-monoacetate was equal to 1). The mobility behavior of acetylated X_2 and 18-hydroxydeoxycorticosterone was that which could be expected from a monoacetate because had the diacetate been formed, a compound much less polar than corticosterone-21-monoacetate would have been obtained. This conclusion is also based on the findings of Pappo (6) that a monoacetate is formed when 18-hydroxydeoxycorticosterone is acetylated as above and yields the 21-monacetate derivative (28).

These findings are at variance with our former report (1) in which it was concluded from equivocal infrared data that no free hydroxyl groups were present on compound X_2 after acetylation with acetic anhydride in pyridine. A hydroxyl band should have been registered in the 3500 cm.$^{-1}$ range of the spectrum had the X_2 acetate KBr prism been made correctly.

DISCUSSION AND CONCLUSION

The identification of X_2 was arrived at firstly by the preparation of a lactone (Fig. 1) obtained as the oxidation product of 18-hydroxydeoxycorticosterone and 18-hydroxyprogesterone. Although the optimal reaction conditions for the oxidation of small amounts of these two compounds were not studied a sufficient quantity of the lactone was obtained to permit the recording of several of its physical constants. These were in accord with those reported by Kahnt *et al.* (9) and those communicated to us by Neher (10) for 18-hydroxy-3-keto-4-etien-20-oic acid 20,18-lactone.

The melting point and infrared spectrum of the lactone obtained as the oxidation product of 18-hydroxyprogesterone in this study differed from that given by a lactone obtained by Pappo (6) after the oxidation of 18-hydroxyprogesterone. This is due to the fact that in the latter instance the oxidation of 18-hydroxyprogesterone was carried out with chromic acid in pyridine (6) and yielded 3,20-diketo-4-pregnen-18-oic acid existing as the tautomeric hydroxy-lactone. On the other hand, oxidation of 18-hydroxyprogesterone with chromic acid in glacial acetic acid, as in the present experiment, yielded in the neutral fraction the 21-demethylated 20,18-lactone. This finding is in accord with that reported by Schmidlin and Wettstein (29) who were able to isolate 11β-acetoxy 18-hydroxy-3-keto-4-etien-20-oic acid 20,18 lactone after oxidizing 11β-acetoxy 18-hydroxyprogesterone with chromic acid in glacial acetic acid. Additional evidence

that the structure ascribed to the lactone isolated in this study is correct was also deduced from the report that reacting 18-hydroxydeoxycorticosterone with lead tetraacetate (6) yielded 18-hydroxy-3-keto-4-etien-20-oic acid 20,18-lactone whose infrared spectrum is in accord with those shown in Figure 1. The significance of the weak and rather broad absorption band at 3500 cm.$^{-1}$ which can be seen (Fig. 1) is unknown at present. There is the possibility that this band was due to traces of water which were not removed when the KBr pellets were prepared.

Final confirmatory evidence making the identification of X_2 unequivocal was obtained with authentic 18-hydroxydeoxycorticosterone. Although slight differences were noted in the infrared spectra of the two substances, possibly due to different polymorphic states of the two compounds, other physical characteristics such as paper chromatographic mobilities of the free and acetylated compounds and the periodate oxidation proudcts of the free compounds in several solvent systems, were identical. The observation that the 1720 cm.$^{-1}$ band was absent from the authentic 18-hydroxydeoxycorticosterone infrared spectrum lends support to the possibility as reported previously (1) that this band shown in the spectrum of X_2 is an artifact. Therefore, in spite of these and other small physical differences observed between X_2 and authentic 18-hydroxydeoxycorticosterone, the main body of evidence presented appears to be consonant with the conclusion that X_2 is 18-hydroxydeoxycorticosterone. With these data therefore, the identification of X_2 has been assured.

Of importance too in the present study, is the fact that 18-hydroxydeoxycorticosterone was derived solely from endogenous precursor(s) such as cholesterol (11, 12) and conclusively shows that this steroid is a natural substance produced by rat adrenal tissue. Evidence which has been obtained by the Ciba group however (9) for the ability of adrenal tissue to produce this steroid from endogenous precursor is equivocal since deoxycorticosterone was added to the beef adrenal perfusion medium.

We have been able to confirm the findings of Birmingham and Kurlents (3) that ACTH brings about an increased *in vitro* biogenesis of X_2 and, in addition, have observed that its production can also be increased by the addition of adenosine-3′,5′-monophosphate and reduced triphosphopyridine nucleotide in *in vitro* incubations (13). Therefore, an analysis of this substance in the rat by the PS reaction could prove useful in physiological studies wherein a response of the adrenal to these or other substances when administered *in vivo*, is determined by an analysis of the peripheral blood corticosterone by fluorimetric techniques (14–18).

The production of this steroid by the rat adrenal gland and its probable elaboration in other species of animals has been recognized (3, 19–25). Its unique property of reacting with the PS reagent without having the 17,21-dihydroxy-20-keto structure in the side chain has made it the subject of efforts directed at elucidating its structure (1, 23, 26). At present, no

suitable explanation can be given why the 18–20 cyclohemiketal grouping brings about a reaction of 18-hydroxydeoxycorticosterone with the phenylhydrazine sulfuric acid reagent of PS. There is no chemical reason to believe that the 18–20 cyclohemiketal grouping should facilitate the formation of an intermediate glyoxal, substances which can be produced with facility by the treatment of α-ketolic steroids with cupric acetate (4). The absence of a 17α-hydroxy group in this compound more than likely precludes a reaction similar to that given by cortisol and cortisone with the PS reagent. It has been postulated that the latter involves the formation of a yellow 3,20- or 3,21-bisphenylhydrazone (27) resulting in the typical well-known absorption peak at or close to 410 mμ. Thus, at present, 18-hydroxydeoxycorticosterone takes rank with other steroids (27) which react with the PS reagent but do not possess the 17,21-dihydroxy-20-keto structure in the side chain.

Finally, it must be emphasized that extreme caution should be exercised in the isolation and handling of this steroid. It is unstable, and especially in the presence of acids dimerisation occurs rapidly (28). This same reasoning (30) should apply to attempts at isolating compound X_1 (1) which has been postulated as being a hydroxylated form of X_2. The latter may very well prove to be 18-hydroxycorticosterone because of its mobility characteristics on paper (1) in addition to the fact that the rat adrenal gland possesses a very active 11β-hydroxylating enzyme system (25).

Addendum

Since submission of the manuscript for publication a steroid isolated from the pooled X_1 zones of paper chromatograms has been found to be 18-hydroxycorticosterone as the cyclic 18–20 hemiketal compound. This conclusion was arrived at by comparing the mobility rates of the free, acetylated and periodate oxidation products of X_1 with those obtained under identical conditions from standard 18-hydroxycorticosterone (a generous gift from Dr. R. Neher).

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STUDIES ON THE STATE OF INSULIN IN BLOOD: "FREE" INSULIN AND INSULIN COMPLEXES IN HUMAN SERA AND THEIR *IN VITRO* BIOLOGICAL PROPERTIES[1]

HARRY N. ANTONIADES, PAUL M. BEIGELMAN, ROBERT B. TRANQUADA AND KARE GUNDERSEN[2]

Protein Foundation Laboratories, Jamaica Plain, Massachusetts; the Departments of Biochemistry and Medicine, University of Southern California, Los Angeles, the Boston Dispensary, and the Department of Medicine, School of Medicine, Tufts University, Boston, Massachusetts

ABSTRACT

Studies are presented on the relative amounts of the protein-bound and the "free" forms of insulin in the sera of non-diabetic subjects. The "bound" form of insulin was extracted from sera by a cationic exchange resin (Na^+ cycle) which adsorbed only the "bound" form of insulin. The insulin-protein complexes (basic protein-insulin complexes) in turn were eluted from the resin by alkali. The resin eluates, representing the "bound" form of insulin, were examined for insulin activity by the rat diaphragm assay in the presence of adipose tissue extracts which, as it has been shown, dissociate insulin from its complex.

The insulin-like activity present in the whole serum is characterized as the "free" portion of insulin. This characterization resulted from earlier studies demonstrating that insulin in its "bound" form is devoid of insulin activity when examined under specified conditions.

It is shown further that incubation of whole diluted serum with adipose tissue extract results in a significant increase of glucose uptake by the intact rat diaphragm and the rat adipose tissues. The increase of glucose uptake under these conditions is attributed to the dissociation of the "bound" insulin of serum by a factor or factors present in the adipose tissue extracts.

These studies indicate again that insulin circulates in the blood in "free" and "bound" forms. These two forms of insulin differ in their biological properties in that the "bound" insulin exerts its biological activity only after dissociation of the insulin complex.

IT HAS been reported that a portion of insulin in blood circulates as a complex with other proteins and, more specifically, basic proteins (1–4). The insulin complex(es) ("bound" insulin) were extracted from blood by the use of a cationic exchange resin (Na^+ cycle) and were eluted from the resin by acid or alkali (4). It was also shown that insulin in its "bound" form was devoid of biological activity when examined *in vitro* by the rat

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diaphragm assay (4–6). Insulin activity was demonstrated in these preparations by dissociation of the insulin from its complex either by centrifugation at pH 10 (5) or by incubation of the insulin complexes with adipose tissue extracts (6).

The present communication reports: I. Studies on the relative amounts of the "bound" and "free" forms of insulin in the sera of non-diabetic subjects; II. Data which show that insulin in its "bound" form in whole sera, as in purified blood preparations, (6) can be dissociated *in vitro* by incubation with extracts from adipose tissue. This results in a marked increase of insulin-like activity over that due to the "free" insulin present in sera.

The following systems of insulin assay have been used in the above studies: I. Determination of the amounts of "bound" and "free" insulin in sera were carried out by the rat diaphragm assay as described by Vallance-Owen and Hurlock (7). Whole undiluted sera were used for the assays, and the results were expressed in micro-units of insulin per milliliter of serum. II. Dissociation of the "bound" form in whole serum by adipose tissue extracts was studied both by the use of a modification of the diaphragm assay (8) and by a modification (9) of the original adipose tissue assay of Martin *et al.* (10) In these studies sera were diluted with buffer before the assay, and the results were expressed in terms of glucose uptake by the rat diaphragm or by the rat adipose tissue.

The "bound" form of insulin was extracted from sera by using acationic exchange resin (Na^+ cycle) which adsorbed only the "bound" form of insulin (4). The insulin-protein complexes, in turn, were eluted from the resin by alkali (4) and were examined for insulin-like activity in the presence of adipose tissue extracts (6). The portion of insulin-like activity assayed directly in the whole serum was considered "free" insulin. This supposition is based on the observation that "bound" insulin is devoid of activity (4–6) when assayed by the rat diaphragm assay used in this study.

The results presented in this communication indicate again that insulin circulates in the blood in "free" and "bound" forms. These two forms of insulin differ in their biological properties, in that the "bound" insulin exerts its biological activity only after dissociation of the insulin complex. It appears as though the "bound" form of insulin in blood serves for the transport of insulin in a biologically inactive form. The storage of insulin in human and bovine pancreas in a complex form with other proteins has been reported elsewhere (11).

MATERIALS AND METHODS

Determination of "Bound" and "Free" Insulin-Like Activity in the Serum of Non-Diabetics.

One hundred to 200 ml. of whole blood were collected from each non-diabetic subject. The blood was allowed to clot, and the serum was separated by centrifugation at 3000 r.p.m. for 30 minutes.

Each serum was passed through a cationic exchange resin (Na+ cycle) column, 3.5×10 cm., containing one-half the serum volume of wet-Dowex-50×8 resin, pH 6.4 ±0.2, at a rate of 10–15 ml. per minute. Following the passage of the serum, the resin column was washed with 1 resin volume of 0.15 M NaCl. The "bound" insulin adsorbed in the resin was eluted with 1 resin volume of 0.1N NH_4OH, followed by a washing with 1 resin volume of cold distilled water. The ammonium hydroxide eluate and the wash were collected under continuous stirring in 1/5 resin volume of 0.2N H_2SO_4, adjusted to pH 2.0, in order to avoid a long exposure of the insulin to a high pH. The final pH was adjusted as soon as possible to 7.2±0.4. The eluate was lyophilized from the frozen state. The dry material was dissolved with distilled water to the volume desired for the assay, usually ½ to ⅓ the original serum volume. The eluates and the sera, after resin treatment, were dialyzed at 2° C for 18 hours against 20 to 30 volumes of Gey and Gey bicarbonate buffer, twice, before the assay. The resin eluates, containing the "bound" insulin, were incubated for 30 minutes at 37° C with rabbit or rat adipose tissue extract and assayed for insulin-like activity as described elsewhere (6).

A portion of the serum before and after passage through the resin column also was assayed for insulin-like activity by the rat diaphragm tissue assay (7). The resin-treated sera were dialyzed overnight against Gey and Gey bicarbonate buffer prior to insulin assay in order to restore lost electrolytes adsorbed on the resin.

Assays for insulin-like activity in the study of the ratio of "bound" and "free" insulin in serum were carried out in triplicate by the rat diaphragm assay as described by Vallance-Owen and Hurlock (7).

Examination of Insulin-Like Activity in Whole Serum in the Presence of Rabbit Adipose Tissue Extracts

Whole serum collected from 2–4 non-diabetic donors was pooled and diluted with Krebs bicarbonate buffer. Dilutions of the pooled sera were assayed for insulin-like activity with and without rabbit adipose tissue extract. Perirenal adipose tissue collected from rabbits was homogenized in Krebs bicarbonate buffer by a Teflon tissue grinder, using the method previously described for the preparation of rat adipose tissue extracts (6). After centrifugation, the supernatant fluid was stored at −20° C. One milliliter of this rabbit adipose tissue extract containing 0.05 to 0.1 mg. of nitrogen was added to 10 ml. of serum dilution to be tested.

Determinations of the glucose uptake in these studies were carried out with modifications of both the rat diaphragm and the rat adipose tissue assay, as described below.

Rat Adipose Tissue Assay (The assay used was a modification by Beigelman (9) of the original assay of Martin *et al.* (10).

Adipose tissue segments (40–50 mg.) of male Wistar rats (120–240 gm.) were used. The incubation medium volume was 2 ml. per beaker. Incubation proceeded for 2–6 hours at 36.5° C under 95% O_2–5% CO_2. Glucose determinations utilizing the Somogyi-Nelson method were performed following incubation. The difference between the glucose content of medium from beakers containing adipose tissue and identical medium from "blank" beakers containing no tissue constituted glucose uptake. The values for glucose uptake per gram of adipose tissue are expressed as the logarithm.

Rat Diaphragm Tissue Assay (8): Non-fasted male Wistar rats weighing 90–150 gm. were stunned by a blow on the head and killed by decapitation. The diaphragm was removed intact, blotted on filter paper, the thick posterior portion removed, and remaining connective tissue and blood vessels were trimmed off. The diaphragm was then quartered, and quarters were placed into 25 ml. flasks containing 2 ml. of medium. The flasks were stoppered and individually gassed for 5 minutes with 95% O_2, 5% CO_2 while being shaken at room temperature (23–27° C). Upon completion of gassing, the incubator

temperature was brought up to 37° C, and the flasks were shaken at a rate of 90 cycles per minute for 90 minutes.

At the end of the incubation period the tissue was removed from the flasks, blotted, dried for 2 hours at 100° C and weighed on a torsion balance. One ml. aliquots were removed from the flasks, precipitated with $Ba(OH)_2$ and $ZnSO_4$, and the glucose concentration was determined by a glucose-oxidase method (12).

The incubation medium consisted of Krebs-Ringer-bicarbonate buffer containing 130 mg.% glucose. Serum dilutions were also performed with this buffer. All incubations included 20 flasks, divided into 4 groups, each group consisting of a non-tissue control and four flasks containing a quarter diaphragm each. Tissue from each of the four rats used per experiment was included in each group of flasks. Glucose utilization was calculated in terms of micrograms glucose utilized per 10 mg. diaphragm (dry weight).

EXPERIMENTAL—RESULTS

"Bound" and "Free" Insulin-Like Activity in Non-Diabetic Sera

The results of the studies on the ratio of "bound" and "free" insulin-like activity are summarized in Table 1. There is a large variation in the ratio of "bound" and "free" insulin-like activity among the individual non-diabetic donors. Following passage of the sera through the resin column, the "free" insulin-like activity remained in the resin-treated sera.

The values reported in Table 1 should be regarded as the minimum amounts of "bound" insulin in serum. Losses of "bound" insulin may result from the effort to adsorb the insulin complexes on the cationic exchange resin (Na^+ cycle) and in turn to elute and concentrate the individual preparations.

Incubation of Whole Serum with Rabbit Adipose Tissue Extracts

Pooled samples of whole serum obtained from non-diabetic subjects 2–5 hours after a meal were employed in these studies. The sera were diluted

TABLE 1. "BOUND" AND "FREE" INSULIN-LIKE ACTIVITY (ILA) IN THE SERA OF NON-DIABETIC SUBJECTS

	Insulin-like activity in μu. per ml. Serum				
Serum No.	Whole serum before resin treatment ("free" ILA)	Resin eluates* plus ATE ("bound" ILA)	Whole serum after resin treatment ("free" ILA)	Total insulin-like activity	Ratio "bound"/ "free"
1	226	194	275	420	0.87
2	475	23	405	498	0.05
3	227	800	—	1027	3.50
4	70	214	160	284	3.00
5	185	122	104	307	0.70
6	12	400	—	412	33
7	64	276	125	340	4
8	< 10	168	10	168	16.80
9	< 10	300	—	300	30
10	342	28	—	370	0.08

* Determinations of insulin-like activity were carried out in the presence of rat or rabbit adipose tissue extracts.

TABLE 2. GLUCOSE UPTAKE BY RAT DIAPHRAGM TISSUE FOLLOWING INCUBATION OF WHOLE SERUM WITH AND WITHOUT RABBIT ADIPOSE TISSUE EXTRACTS* (ATE)

Pooled sera†	No. of assays	Mean glucose uptake in μg/10 mg. dry diaphragm ±S.E.			"p" Comparison of serum plus ATE with	
		Serum alone	Serum plus ATE	Control uptake	Serum alone	Buffer control plus ATE
Pool II‡	16	102 ± 7§	283 ± 8		< .01	< .01
Pool III‡	19	65 ± 5	162 ± 6		< .01	< .01
Pool IV‡	20	25 ± 8	169 ± 8		< .01	< .01
Pool V‡	4	69 ± 7	154 ± 8		> .02, < .05	< .01
ATE alone	15			23 ± 5		
Krebs Buffer alone	34			154 ± 11		

* 0.05 to 0.1 mg. nitrogen per ml. incubating medium.
† Pooled non-diabetic sera (3 to 4 donors).
‡ All sera were diluted 1/30 with Krebs bicarbonate buffer.
§ Calculated as net glucose uptake above control (buffer alone).

with Krebs bicarbonate buffer and were assayed with or without the presence of rabbit adipose tissue extract the same day. One milliliter of rabbit adipose tissue containing 0.05 to 0.1 mg. of nitrogen was added to 10 ml. of incubating medium.

Determinations of the glucose uptake in these studies were carried out both with a modification of the rat diaphragm tissue assay (8) and with the adipose tissue assay (9).

Results Obtained by the Rat Diaphragm Assay: The results of these experiments are summarized in Table 2. A significant increase in glucose uptake was observed with each serum when incubated with rabbit adipose tissue extract. The increase in glucose uptake depends on the concentration of the adipose tissue extract as is shown in Table 3 and Figure 1. Figure 1 also demonstrates the similar relationship of the concentration of rabbit adipose tissue extract to the glucose uptake of diluted serum and that of rat adipose tissue extract to the glucose uptake of purified insulin complexes extracted from serum with cationic exchange resin (Na^+ cycle) (6).

Storage of the crude rabbit adipose tissue extract at 5° C for 18 hours resulted in a significant loss of activity (Table 4). Loss of activity during

TABLE 3. THE EFFECT OF DILUTION ON THE ACTIVITY OF RABBIT ADIPOSE TISSUE EXTRACT (ATE)

	Serum alone	Serum* plus rabbit ATE in μg. nitrogen per ml. incubating medium				
		1 μg	3.3 μg	10 μg	20 μg	100 μg
Mean glucose uptake in μg/ 10 mg. dry diaphragm ±S. E.	36 ± 10†	37 ± 3	88 ± 6	103 ± 6	130 ± 3	155 ± 5
No. of assays	15	8	8	16	8	8

* Diluted 1/30 with Krebs bicarbonate buffer.
† Calculated as net glucose uptake above control (buffer alone).

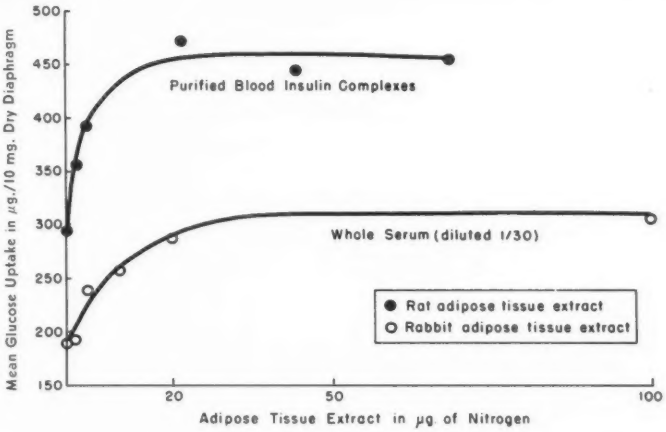

FIG. 1. Glucose uptake by rat diaphragm following incubation of purified blood insulin complexes* and diluted serum** with various concentrations of adipose tissue extracts. The protein concentration of the incubating medium is approximately 2 mg per ml. medium for the diluted serum and 0.18 mg. per ml. medium for the purified blood insulin complexes. One ml. of the purified preparation represents about 4 ml. of undiluted serum.

* Determinations of glucose uptake were made by the rat diaphragm assay as described by Vallance-Owen and Hurlock.

** Determinations of glucose uptake were made by a modification of the rat diaphragm assay by Tranquada (8).

dialysis at 5° C for 18 hours could be attributed to instability of the extract under these conditions.

Results Obtained by the Rat Adipose Tissue Assay: The insulin-like activity of whole serum is significantly increased in the presence of rabbit adipose tissue extracts, as is shown in Table 5.

These results are of particular interest since it was shown in previous studies (4–6) that intact adipose tissue could utilize insulin in its complexed form. The amount of "bound" insulin thus utilized will depend upon the conditions of the assay, such as the weight and the surface area of the

TABLE 4. THE EFFECT OF STORAGE (5° C) AND DIALYSIS (5° C) ON THE ACTIVITY OF RABBIT ADIPOSE TISSUE EXTRACT

	Serum* alone	Serum* ATE	Serum plus stored ATE†	Serum plus dialyzed ATE‡
Mean glucose uptake in μg/10 mg. dry diaphragm ± S.E.	34 ± 6§	223 ± 5	58 ± 7	64 ± 6
No. of assays	8	8	8	8

* Diluted 1/30 with Krebs bicarbonate buffer.
† Stored at 5° C for 18 hours.
‡ Dialyzed against Krebs bicarbonate buffer at 5° C for 18 hours.
§ Calculated as net glucose uptake above control (buffer alone).

TABLE 5. GLUCOSE UPTAKE BY RAT ADIPOSE TISSUE FOLLOWING INCUBATION OF WHOLE SERUM WITH AND WITHOUT RABBIT ADIPOSE TISSUE EXTRACTS* (ATE)

Pooled sera†	Dilution	Glucose uptake (log 10 glucose uptake per gm. adipose tissue)								
		Duration of incubation								
		4 hours			5 hours			6 hours		
		No. of assays	Glucose uptake ± S.E.	"P"‡	No. of assays	Glucose uptake ± S.E.	"P"‡	No. of assays	Glucose uptake ± S.E.	"P"‡
Pool II + III alone	1/30				18	.663 ± .020	> .05, < .1	20	.860 ± .015	> .02, < .05
Plus ATE					20	.804 ± .015		20	1.039 ± .040	
Pool IV alone	1/30	19	.600 ± .052	< .01	6	.912 ± .045	> .02, < .05			
Plus ATE		18	.747 ± .051		6	1.041 ± .033				
Pool V alone	1/30	4	.363 ± .065	> .02, < .05				16	.813 ± .050	
Plus ATE		4	.630 ± .070					16	.969 ± .055	> .02, < .05
Pool VI alone	1/30	13	.559 ± 0.06	< .01						
Plus ATE		13	.787 ± 0.04							
Buffer alone		6	.616 ± .056		14	.653 ± .080		24	.890 ± .035	
Plus ATE		4	.402 ± .137		15	.623 ± .055		29	.887 ± .050	

* 0.05 to 0.2 mg. nitrogen per ml. incubating medium.
† Pooled non-diabetic sera (3 to 4 donors).
‡ Comparison of serum plus ATE with serum alone.

adipose tissue, the time of incubation and the concentration of "bound" insulin. It is possible therefore, as suggested from the results of Table 5, that the intact adipose tissue could utilize only part of the "bound" insulin, the amount depending upon the conditions of the assay. Incubation for four hours yielded higher differences between the control serum and serum plus rabbit adipose tissue extract than incubation for six hours. The less significant differences observed after six hours incubation can be attributed to utilization by the intact tissue of a larger portion of the "bound" insulin in the control serum.

Rabbit adipose tissue extracts heated for one hour at 100° C showed a significant loss of activity (Table 6). It appears that the components

TABLE 6. THE EFFECT OF HEATING ON THE ACTIVITY OF RABBIT ADIPOSE TISSUE EXTRACT (ATE)*

	Serum† alone	Serum plus ATE	Serum plus heated ATE‡	"P" Serum alone compared with serum plus ATE	"P" Serum alone compared with serum plus heated ATE
Log 10 glucose uptake per gm. adipose tissue	.559 ± .06	.787 ± .04	.666 ± .045	< .01	> .05, < .1
No. of assays	13	13	14		

* 4 hour incubation with rat adipose tissue.
† Diluted 1/30 with Krebs bicarbonate buffer.
‡ Heated at 100° C for 1 hour.

responsible for the activity of crude adipose tissue extracts are sensitive both to storage (Table 4) and to heating at 100° C (Table 6).

DISCUSSION

In a previous communication it was shown that incubation of purified blood insulin complexes with adipose tissue extracts of various species resulted in an increase of insulin-like activity in these extracts (6). Identical insulin complexes assayed in the absence of adipose tissue extracts were either devoid of insulin-like activity or demonstrated low insulin-like activity as compared with the activity obtained in the presence of the adipose tissue extract. Control experiments showed that adipose tissue extracts alone were devoid of insulin-like activity. Adipose tissue extracts added to crystalline insulin did not alter the activity of commercial "free" insulin (6).

The increase of insulin activity in purified blood insulin complexes in the presence of the adipose tissue extracts was attributed to the release of insulin from its complex form through the intervention of a factor or factors present in the adipose tissue extracts. The similarity between the results obtained with whole serum in the presence of adipose tissue extracts and the results obtained with the purified insulin complexes suggests that

addition of the adipose tissue extract to the serum results in liberation of the "bound" insulin present in serum.

The effect of the adipose tissue extracts appears to be related to the concentration of the extract and the duration of incubation. The mechanism of the liberation of insulin from its bound form in the presence of adipose tissue extracts is not known. It may be due to destruction of the protein which transports the insulin, the dissociation of the insulin from this protein or other mechanisms thus far unknown.

Crude rabbit adipose tissue extracts stored at 5° C for 18 hours or heated at 100° C for one hour showed loss of activity. It appears that the factors responsible for the adipose tissue extract activity are sensitive to storage and heating. These experiments were performed with crude extracts. One may speculate that if the active component in this crude extract is of a protein nature, loss of activity by storage may be due to proteolytic enzymes or bacterials present in the extract. This suggestion is supported by the observation that partially purified rat adipose tissue extract (60% ethanol supernatant) subjected to paper electrophoresis for 18 hours at 5° C did not lose its activity (6).

The increase in insulin-like activity of serum in the presence of adipose tissue extract will depend upon the ratio of "free" to "bound" insulin in serum. Our studies indicate considerable variation in "bound" insulin concentration among non-diabetic subjects. This ratio does not seem to represent a purely physico-chemical equilibrium between "bound" and "free" insulin. The rate of dissociation of "bound" insulin in blood could be affected by other factors such as those present in adipose tissue extracts. Recent experiments suggest that a rise of blood glucose may "trigger" a mechanism which results in the dissociation of the "bound" insulin (13).

These considerations suggest that the transport of insulin in blood by other proteins may serve both to regulate insulin activity and to provide for the storage of circulating insulin in a biologically inactive form.

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THE EFFECT OF AGE AND THYROID HORMONES ON THE MONOAMINE OXIDASE OF RAT HEART

WILLIAM J. NOVICK, JR.[1]

Department of Physiology and Pharmacology, Duke University Medical Center, Durham, North Carolina

ABSTRACT

The activity of monoamine oxidase was studied in the heart mitochondria of normal and hyperthyroid rats using manometric techniques. A definite age effect was observed in normal rats with an increase from 0-trace activity in post-natal to approximately 1000 μl. O_2 consumed/gram/hour in 5–600 gram rats. Desiccated thyroid diet (2%) and L-triiodothyronine produced an increase in heart monoamine oxidase activity of from 70 to 200% depending upon the age group of the animals used. D-Triiodothyronine did not have this stimulating effect when used at equimolar doses. The effect of oxygen tension on the oxidation rate of tyramine is different for the heart and liver enzymes.

THYROID hormone causes a number of changes in enzyme activity (1). In particular, Zile and Lardy produced a decrease in rat liver monoamine oxidase (MAO) by feeding desiccated thyroid (2). Zile further reported the same results using thyroxine and several thyromimetic compounds (3). Zile also reported that these thyromimetic compounds produced no measureable change in heart MAO (3).

The present study does not fully support the findings of Zile. We obtained significant increases in heart MAO after administration of L-triiodothyronine (LT3) (Smith, Kline & French Laboratories) or desiccated thyroid diet (2%). There was also an increase in MAO activity of the heart from the time of weaning to old age. The effect of LT3 was greater in the younger animals which had a normally low MAO activity. The increase in heart MAO was not produced with equivalent doses of d-triiodothyronine (DT3) (Smith, Kline & French Laboratories).

It was also found that the oxidation rate of tyramine by the heart enzyme was doubled when the oxygen tension was raised from 20% to 100% but that of liver was tripled.

METHODS AND MATERIALS

The source of enzyme was washed mitochondria prepared according to Schneider (4). Rats (Duke colony of Osborn & Mendle strain) were killed by a blow on the head, decapitated and the heart and liver were removed and placed in crushed ice. After the tissues were cooled, blotted dry, and weighed, they were minced with scissors and placed in cold sucrose (0.25 M.). The liver was homogenized in a Potter-Elvehjem homogenizer at a $\frac{1}{10}$ to $\frac{1}{15}$ dilution. Heart was homogenized first in a Waring Blender using cold

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[1] Walter Karr Fellow. Smith, Kline & French Laboratories, Philadelphia, Pennsylvania.

sucrose and then was homogenized in a Potter-Elvehjem homogenizer. Both homogenates were centrifuged at 600×g. for 15 minutes at 3–4° C in an International Refrigerated Centrifuge. This step was repeated and the combined supernates were then spun at 16,000×g. for 20 minutes in a Servall Superspeed Centrifuge kept at 4–8° C. This step was also repeated and the pellets were taken up in 0.067 M. Na-phosphate buffer, pH 7.4. The final volume was such that 2.0 cc. contained the equivalent of 250–300 mg. fresh weight tissue. In the case of young animals, several hearts were combined.

The reaction mixture consisted of 2.0 cc. of homogenate, 20 μM semicarbazide in 0.2 cc. buffer and 0.3 cc. of substrate contained in the side arm of a Warburg flask. The measurement of oxygen uptake followed standard manometric techniques. All assays were carried out at 37° C using air or 100% O_2 as the gas phase. Each sample was also assayed for endogenous O_2 uptake.

The use of semicarbazide in this system has been shown to result in stoichiometric agreement between NH_3 produced and O_2 consumed (5). Semicarbazide acts as a trap for the aldehyde and prevents further oxidation of this product. We have shown that NH_3 production (6) and O_2 consumption are in agreement in our system if air or 100% O_2 is used as the gas phase.

The activities are reported as μl. O_2 consumed/gram fresh weight tissue/hour calculated from the O_2 uptake during the first 15 min. The specificity of the assay method was checked by producing 100% inhibition of oxygen uptake with 2×10^{-4} M. iproniozide (Hoffman-LaRoche, Inc.) as well as 2×10^{-5} M. 2-phenylcyclopropylamine (Smith, Kline & French #385). It was further shown that 97–99% of the expected O_2 uptake based on added substrate was obtained in this system. Tyramine hydrochloride at 20 μM per flask was used as the substrate.

RESULTS

Effect of Oxygen Tension

An increase in the rate of MAO activity with increased oxygen tension has been reported (7). In the present study, a 3.44 fold rise in activity of liver MAO was found when the gas phase was changed from air to 100% O_2 (average of 30 assays for each gas phase). However, heart enzyme was found to have only a 1.96 fold rise under the same conditions (average of 13 assays). The oxygen sensitivity of heart MAO was not a function of age as is shown in Table 1.

Effect of Age

Heart has been reported as having very low MAO activity (8). It was observed early in this study that differences in MAO activity of heart were

TABLE 1. EFFECT OF O_2 TENSION ON MAO ACTIVITY IN HEART AND LIVER

	μl. O_2/gm./hr.		Increase (100% O_2/air)
	Air	100% O_2	
Liver (av. 30 animals)	512	1760	3.44
Heart 100 gm. Rat	65	135	2.08
Heart 150 gm. Rat	135	270	2.00
Heart 260 gm. Rat	224	436	1.95
Heart 520 gm. Rat	517	1095	2.12
Heart 610 gm. Rat	580	979	1.70

obtained in various groups of animals. The highest values were from the heaviest weight group, thus a survey of heart MAO was undertaken from newborn to 600 gram rats. These data are graphically represented in Figure 1. No MAO activity could be detected in the hearts of new born

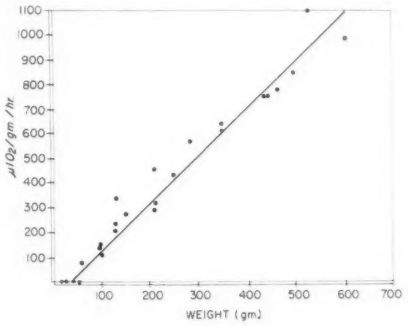

FIG. 1. Heart MAO activity as a function of age (weight). Each point represents at least one animal. Tyramine was used as the substrate and 100% O_2 as the gas phase. The solid line is for reference only and was drawn by eye.

rats (1–2 day old) even though 400 mg. samples were assayed. In some cases, homogenates rather than mitochondria were used and the same negative results were obtained. This lack of activity remained true for all groups of animals up to 3 weeks of age. MAO activity was first detected by our method at about 4 weeks and continued to rise throughout the life of the rat reaching a level of about 1,000 μl.O_2/gm./hr. at the maximum weight tried (610 gm.). This is in the range of very active tissues such as liver which has an activity of about 1,700 μl. O_2/gm./hr. under similar conditions (Table 1).

Hyperthyroid Studies

Animals were separated into three age groups: 1) adult rats in the range of 200 gm.; 2) young rats from weaning to 100 gm. and 3) postnatal to weaning rats. All groups were given LT3 (150 μg/kg./day sub.c.) or DT3 (150 μg/kg./day sub.c.) for a period of 8–10 days. (Group 3 received the drugs for 15 days.) A group of adult rats was placed on a 2% desiccated thyroid diet for 12 days. All animals were sacrificed at the termination of drug administration. Heart MAO was assayed using tyramine as a substrate and 100% O_2 as the gas phase. The results are listed in Table 2.

The degree of hyperthyroidism was shown by a 100% increase in BMR over control animals when LT3 was administered for 6 days. Adult rats receiving the 2% desiccated thyroid diet had a BMR of 30% higher than control animals at the same period of treatment.

Adult rats showed a small but significant increase in heart MAO after administration of LT3 (70% above control level). However, DT3 failed to produce a similar increase and the DT3 animals remained at control

TABLE 2. HEART MAO ACTIVITY IN THE HYPERTHYROID STATE

Each assay represents at least one animal. The % increase was calculated by: (Experimental − Control) ÷ Control × 100.

Group	Drug	No. of assays	Days treated	μl. O_2/gm./hr.	% increase
Adult (200 gm.)	Control	5	—	293 ± 62	
Adult (200 gm.)	LT3	16	8	497 ± 84	70%
Adult (200 gm.)	DT3	5	8	303 ± 45	
Adult (250 gm.)	Control	5		454 ± 48	
Adult (250 gm.)	Desiccated Thyroid	8	12	794 ± 96	75%
Young (<100 gm.)	Control	6	—	52 ± 20	
Young (<100 gm.)	LT3	9	8	158 ± 47	204%
Young (<100 gm.)	DT3	3	8	60 ± 22	
Neonatal	Control	5	—	0 trace	
Neonatal	LT3	6	15	187 ± 42	
Neonatal	DT3	3	15	0 trace	

levels. Desiccated thyroid diet produced an increase in heart MAO activity equivalent to the LT3 study (75% increase).

Young rats (<100 gram) showed a greater increase in heart MAO after administration of LT3 (200% increase) than the adult animals. Again, DT3 failed to produce any change in heart MAO. No attempt was made to feed this group desiccated thyroid.

The most dramatic change in heart MAO was obtained in the postnatal group. Control animals at this age have no heart MAO activity or, at best, just a trace amount. However, after LT3 was administered from the 7th to the 21st day of life, the heart MAO activity had increased to a level equivalent to a 170–180 gram control rat. DT3 was also found to give negative results in this age group.

Liver MAO was assayed in the adult animals receiving a 2% desiccated thyroid diet. After 12 days of treatment, the activity of liver MAO was only 50% that of control animals using tyramine as substrate in air or 100% O_2. This is in agreement with the results of Zile and Lardy (2).

DISCUSSION

The stimulation of MAO by increased oxygen tension is an interesting phenomenon which is yet to be explained. This increase in activity is not the same for all substrates. For example, serotonin gives only a 1.4 fold increase with the liver enzyme when the gas phase is changed from air to 100% O_2 (9). The difference in the oxygen effect between the liver and the heart enzymes when the same substrate was used indicate that the MAO characteristics are not the same for these two tissues. Perhaps this may help to explain the opposite effects of thyroid hormone on liver and heart MAO as well as the apparent preferential inhibition of MAO in certain tissues by inhibitors of this enzyme (10).

Many examples have been given of the lack of certain enzymes at birth (9), but these usually appear shortly after birth and rapidly rise to the adult level. The lack of MAO activity in heart for the first three weeks of

life is longer than that observed with most other enzymes. A possible clue to the initiation of enzyme production in heart is that it appears shortly after weaning.

The increase of heart MAO during the life of the rat is not unusual. Other enzymes have been observed to follow the same pattern in liver (11). It may be of interest, however, that an enzyme such as MAO which plays a role in the catabolism of catechol amines (12) should have a continuously increasing level in heart.

The stimulation of heart MAO by the hyperthyroid state cannot be explained at this time. That thyroid hormones exert this effect is suggested by the observation that thyroidectomy produced a 50% decrease in heart MAO activity in rats (13).

Heart undergoes several basic changes in the hyperthyroid condition which include metabolic as well as physical changes. The increase in MAO may be due to a direct effect of thyroid hormone to increase the synthesis of heart MAO. However, it may be an indirect effect of the hypertrophy caused by the increased cardiac output. It would be interesting to see if hypertrophy caused by other means would also cause an increase in heart MAO activity.

As mentioned earlier, our results do not agree completely with those of Zile (3). This might be explained by two factors. Their assay method used air as the gas phase and thus the measured activities would be only one half of those in our study. With the low levels of MAO activity in heart, this would lead to difficulties in determining small differences from the control. They also used rats of the 225–250 gm. range which we have shown to be relatively insensitive to changes in heart MAO due to administration of LT3.

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PHYSIOLOGICAL AND RADIOISOTOPIC EVIDENCE FOR THE BINDING OF HYDROCORTISONE BY HUMAN LEUCOCYTES *IN VITRO*[1]

MELVIN M. KETCHEL

Department of Surgery, Harvard Medical School and the Peter Bent Brigham Hospital, Boston and the Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts

ABSTRACT

The ameboid migration of human leucocytes, as measured in capillary tubes, is inhibited by the addition of 10 μg/ml. of hydrocortisone. If the leucocytes are incubated with 10 μg/ml. hydrocortisone, washed, and then put into plasma to which no hydrocortisone has been added, the inhibition still occurs. If leucocytes are incubated with 10 μg/ml. of hydrocortisone, washed, and then put in plasma to which 10 μg/ml. of hydrocortisone is added, no more inhibition occurs than if the hydrocortisone were present only during the incubation or only during the migration period. A period of 5 minutes is sufficient for the incubation with hydrocortisone to cause an inhibition in leucocyte migration. Incubation of leucocytes with the hydrocortisone is effective in a non-protein salt-solution, as well as in plasma. This physiological evidence of the binding of hydrocortisone by leucocytes *in vitro* is substantiated by the detection of radioactivity in leucocytes after incubation with hydrocortisone-4-C^{14}.

THE ameboid migration of human leucocytes in capillary tubes has been shown to be inhibited by relatively low concentrations of hydrocortisone (1). Several lines of reasoning suggest that the hydrocortisone is acting in its capacity as a hormone, rather than in a non-specific manner. These reasons are (1) steroids with similar chemical structures which have no known intrinsic biological activity or activity of a different nature, such as tetrahydrocortisone and desoxycorticosterone, were not effective; (2) hydrocortisone was effective in relatively low concentrations (1–10 μg/ml.); and (3) hydrocortisone from several manufacturers, and in either the free form or as the sodium succinate derivative, was effective, thus decreasing the possibility that a contaminant in the hydrocortisone could be responsible for the effect.

The observation that hydrocortisone inhibited the ameboid migration

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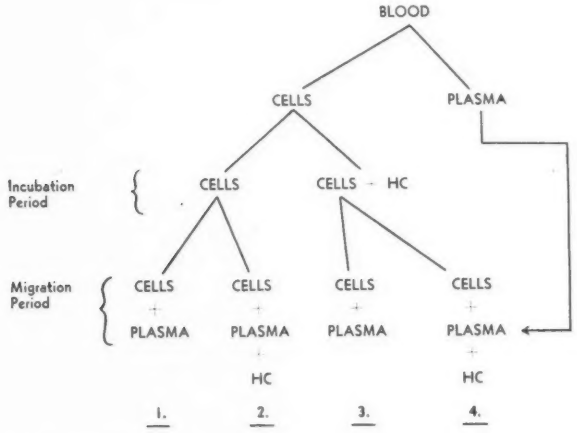
of leucocytes was made on leucocytes migrating in plasma to which hydrocortisone had been added. In an attempt to gain some understanding of the mechanism by which a hormone affects a complicated physiological process such as ameboid motion, a further series of experiments has been performed to answer the following specific question. Must the leucocytes be in the presence of the hydrocortisone while they are migrating in order to be affected, or will a previous exposure to hydrocortisone, before the leucocytes begin to migrate, be effective? Preliminary results (2) are extended and amplified in the present report.

MATERIALS AND METHODS

The design of the present experiments is outlined in Figure 1. Ten ml. of heparinized human blood was separated into cells and plasma. The cells were washed twice in Hanks's



1. Controls—no hydrocortisone (HC).
2. HC during migration only.
3. HC during incubation but not during migration.
4. HC both during incubation and during migration.

FIG. 1. For explanation see text.

solution (3), divided into two equal aliquots, and resuspended in plasma. Hydrocortisone sodium succinate[2] to make a final concentration of 10 μg/ml. was added to one aliquot. After allowing the cells to incubate with the hydrocortisone for specific periods of time, both aliquots were washed twice in 12 volumes of Hanks's solution. Thus, except for hydrocortisone which might have been bound to the cells, only traces of hydrocortisone remained. One-half the aliquot which had been incubated without hydrocortisone was added to plasma to which no hydrocortisone had been added. The other half of the same aliquot was added to plasma to which hydrocortisone had been added to make a final concentration of 10 μg/ml. This procedure was repeated for the aliquot which had been

[2] Solu-Cortef, Upjohn Company, Kalamazoo, Michigan.

incubated with hydrocortisone. Thus, cells subjected to four different treatments were obtained: (1) cells which had received no hydrocortisone, to serve as controls; (2) cells which received no hydrocortisone during the incubation period, but which were in plasma with added hydrocortisone; (3) cells which had received hydrocortisone during the incubation period, but which were in plasma to which no hydrocortisone had been added; and (4) cells which received hydrocortisone during the incubation period, and were in plasma to which hydrocortisone had been added. Another control vial, the same as in (1), was prepared, in order to provide a statistical measure of the internal variation in the experiments.

The preparation of the capillary tubes is described in detail in a previous report (4) The capillary tubes were filled for about $\frac{2}{3}$ of their length by immersing the ends of the tubes in the cell suspension. The unfilled ends of the capillary tubes were sealed by a flame, and the tubes were centrifuged toward the sealed ends. This procedure formed a microhematocrit, in which there was a lower layer of packed red cells, an intermediate "buffy coat" of leucocytes, and an uppermost layer of plasma. The tubes were incubated in a vertical position at 37° C overnight, and the leucocytes migrated, by active ameboid motion, through the interstices of the clotted plasma. The distance which the forward boundary of moving leucocytes migrated from the original buffy coat was measured by means of an ocular micrometer in a microscope. For each cell suspension, 10 capillary tubes were prepared, and an average value taken. This method measures the fastest cells in the population, which are found to be polymorphonuclear cells.

RESULTS

The results of a series of experiments outlined in Figure 1 are shown in Figure 2. The cells that migrated in the presence of added hydrocortisone were inhibited in their migration by an average of 22%. Those cells which had been incubated with hydrocortisone before migration, but which had migrated in the absence of added hydrocortisone, were inhibited by an average of 15%. Those cells which had been incubated with hydrocortisone before migration, and which also had added hydrocortisone present during migration, showed an inhibition of 18%. Thus, those cells treated with

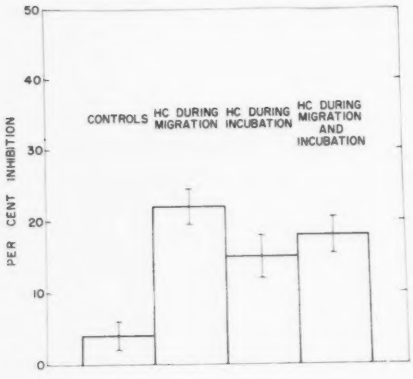


FIG. 2. Comparison of the effect on leucocyte migration of treatment with hydrocortisone before migration, during migration, and both before and during migration. In this and the following figures, the bar labelled controls represents a second set of controls included to provide a statistical measure of the inherent variability of the method. The height of the thin vertical line through each mean represents 2 standard deviations. Leucocytes treated with hydrocortisone during the incubation period, during the migration period, or both, were significantly inhibited in their migration as compared to the controls (0.01 level), but were not significantly different from each other.

hydrocortisone either during the incubation period, during the migration period, or both, were significantly inhibited in their migration as compared to the controls (0.01 level), but were not significantly different from each other. It appears that the leucocytes take up hydrocortisone during the incubation with hydrocortisone, or that the leucocytes are altered in some manner by the incubation with hydrocortisone, so that their migration is affected in plasma to which no hydrocortisone is added. Further, the addition of more hydrocortisone (at the same concentration) to the plasma does not further inhibit the migration of the leucocytes which have been previously incubated with hydrocortisone.

The results shown in Figure 2 were obtained in experiments in which the cells were incubated with hydrocortisone for two hours before being washed

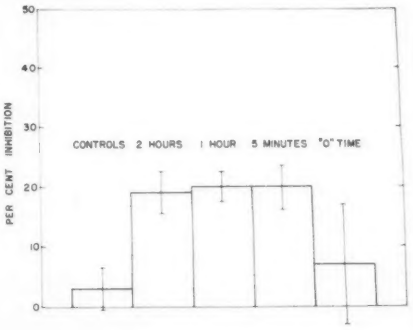


FIG. 3. Incubation with hydrocortisone for 2 hours, 1 hour, or 5 minutes causes a significant inhibition of leucocyte migration as compared to the controls (0.01 level).

and added to plasma for preparation of capillary tubes. Figure 3 shows the results of a series of experiments in which the incubation period was reduced. Since the hydrocortisone concentration is reduced by the addition of wash solution, the time required for centrifugation is not included. It can be seen in Figure 3 that either 1 hour or 5 minutes of incubation with hydrocortisone provided as much inhibition of leucocyte migration as two hours. However, when the wash solution was added immediately after the cells were suspended in hydrocortisone (less than 5 seconds), a very large standard deviation occurred. In some experiments inhibition occurred, but in others it did not. Nevertheless, it seems clear that a period of time as short as 5 minutes is ample for the leucocytes to take up the hydrocortisone.

In the prevous experiments, the cells were incubated with hydrocortisone in a plasma medium. While the leucocytes will not show ameboid movements in a non-protein medium, it was of interest to know if the leucocytes were able to react with hydrocortisone in a non-protein medium. Therefore, a series of experiments was carried out in which one aliquot of a cell suspension was incubated in plasma with added hydrocortisone, and one aliquot was incubated in Hanks's solution with added hydrocortisone.

After incubation both aliquots were washed with Hanks's solution and returned to plasma for the preparation of capillary tubes. As can be seen in Figure 4, although leucocyte migration was inhibited somewhat less in those cells incubated with hydrocortisone in Hanks's solution as compared to those incubated with hydrocortisone in plasma, a statistically significant inhibition (0.01 level) occurred in Hanks's solution when compared to the controls, to which no hydrocortisone had been added. Thus it appears that the hydrocortisone does not need to be in a protein medium in order to react with the leucocytes.

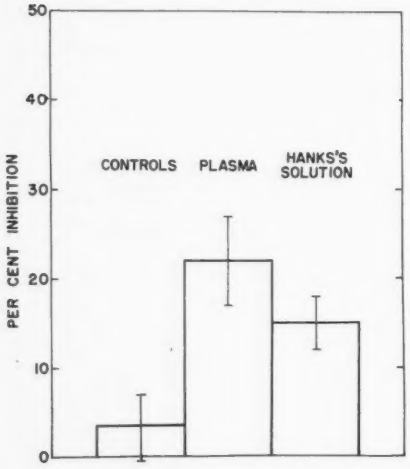


FIG. 4. Significant inhibition of leucocyte migration as compared to the controls is observed when the leucocytes are incubated with hydrocortisone in plasma (0.01 level), or a non-protien salt solution (0.01 level). The difference between plasma and Hanks's solution is not statistically significant.

Although other explanations are possible, the foregoing evidence suggests (1) that the leucocytes take up hydrocortisone to which they are exposed, (2) that the hydrocortisone that is taken up may not be easily washed away from the leucocytes, and (3) that the hydrocortisone so taken up affects a physiological activity of the leucocytes. In order to determine if the leucocytes do take up hydrocortisone, the following experiment was performed. The red cells from 10 ml. of heparinized human blood were sedimented by dextran, and the leucocytes were recovered from the plasma. After being washed 2 times in Hanks's solution, one-third of the leucocytes, in a volume of 0.1 ml., was added to 0.9 ml. of Hanks's solution containing 10 μg of hydrocortisone-4-C^{14}. After incubation at room temperature for 20 minutes, the leucocytes were washed twice in Hanks's solution. The leucocytes were then lysed by addition of distilled water, and placed on a planchet, and dried. A blank, containing everything but the leucocytes was used to determine how much C^{14} not bound to leucocytes was being measured. The difference between the amount of C^{14} in this latter blank, and the amount of C^{14} which occurred in the planchet containing leucocytes was

considered to give the amount of C^{14} taken up by the leucocytes. Another planchet was prepared which contained the same amount of hydrocortisone-4-C^{14} as was incubated with the leucocytes. Thus, the amount of C^{14} in the leucocytes times 100 divided by the amount of C^{14} in 10 μg of hydrocortisone-4-C^{14} gave the percentage of the total hydrocortisone incubated with the leucocytes that was taken up by the leucocytes. In a series of three experiments, 2.76%, 2.37%, and 2.10% of the hydrocortisone was taken up by the leucocytes from the equivalent of 3.3 ml. of peripheral human blood.

Leucocytes recovered from the dextran sedimented blood are not completely free of erythrocytes. In order to determine that the red cells contaminating the leucocyte suspension were not responsible for the measured uptake of hydrocortisone-4-C^{14}, a suspension of erythrocytes, only slightly contaminated by leucocytes, was treated in the same manner as described above for leucocytes contaminated with erythrocytes. In this experiment, only 0.43% of the total hydrocortisone was contained in the cells, indicating that while erythrocytes do bind some hydrocortisone, the leucocytes bind relatively more of it.

A further experiment was performed[3] to ascertain that the C^{14} taken up by the leucocytes was indeed due to hydrocortisone-4-C^{14} and not to non-specific radioactive contaminants. Fifteen μg of hydrocortisone-4-C^{14} was chromatographed on paper using the Bush B5 system (5). After drying the chromatogram, the spot corresponding to known hydrocortisone was eluted, and incubated with leucocytes. Fifteen μg of unchromatographed hydrocortisone-4-C^{14} was incubated with an aliquot of the same leucocyte suspension. The uptake by the two leucocyte suspensions was 0.92% for the chromatographed hydrocortisone-4-C^{14} and 0.62% for the non-chromatographed hydorcortisone-4-C^{14}. A repeat of this experiment gave results of 0.66% and 0.62% for the chromatographed and unchromatographed hydrocortisone-4-C^{14}, respectively. It was therefore concluded that pure radioactive hydrocortisone was being added to the plasma and that either this hormone or a non-specific metabolite of hydrocortisone was being bound by the leucocytes.

DISCUSSION

In the present experiments it has been shown that hydrocortisone need not be in the medium in which the leucocytes are migrating in order to inhibit their migration, provided that the leucocytes have had a previous exposure to hydrocortisone. Further, it has been shown that leucocytes take up hydorcortisone-4-C^{14} from the medium, and this hormone is not removed by washing. It therefore appears that the leucocyte is able to take

[3] I wish to express my indebtedness to Dr. Fernand G. Peron of the Worcester Foundation for Experimental Biology, who carried out the chromatographic procedures and advised me on this experiment.

up hydorcortisone from the surrounding medium, and that this hydrocortisone is able to affect ameboid migration for a considerable period of time afterwards. If these results may be extended to the situation that occurs *in vivo*, it would appear that during periods when the hydrocortisone level in the plasma is low leucocytes and perhaps other cells may be under the influence of hydrocortisone which was bound during times when the plasma concentration of hydrocortisone was high. Thus, if one considers the variations that occur in hydrocortisone levels in the plasma of an individual, it would appear that the most meaningful level, as far as effect on the cells is concerned, would be the highest levels which occur, even though these high levels may be fairly transient.

Acknowledgments

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INTERACTION BETWEEN PLASMA CORTICOSTERONE CONCENTRATION AND ADRENOCORTICOTROPIN-RELEASING STIMULI IN THE RAT: EVIDENCE FOR THE RESET OF AN ENDOCRINE FEEDBACK CONTROL[1,2]

F. E. YATES[3], S. E. LEEMAN[4], D. W. GLENISTER[5] AND M. F. DALLMAN

Department of Physiology, Harvard Medical School, Boston, Massachusetts, and Department of Biochemistry, Brandeis University, Waltham, Massachusetts

ABSTRACT

Experiments were performed to test the hypothesis that the rapid rises in plasma corticosteroid concentration which follow many varied stimuli are caused by a reset of the negative feedback control of plasma corticosteroid concentration to regulate at higher levels. An intravenous dose of histamine, which when given alone produced after 15 minutes an increment in plasma corticosterone concentration of 20 μg/100 ml., was injected 15–30 seconds after an intravenous dose of corticosterone (12 μg/100 gm. B.W.) selected to produce the same increment. The resulting increment in plasma corticosterone concentration equalled that produced by the corticosterone alone: the exogenous corticosterone completely prevented the increased secretion of corticosterone ordinarily provoked by histamine. In a similar fashion, doses of corticosterone (5, 12, and 35 μg/100 gm. B.W.) were selected to produce increments in plasma corticosterone concentration less (10 and 18 μg/100 ml.) and greater (39 μg/100 ml.) than that observed after laparotomy (25 μg/100 ml.). When either of the smaller doses was injected 15–30 seconds prior to laparotomy, the resulting increment equalled, but did not exceed, that observed after laparotomy alone. When the highest dose of corticosterone was injected just prior to laparotomy, the resulting increment in plasma corticosterone concentration equalled that seen after corticosterone alone. These results are those predicted by the reset hypothesis, which provides a basis for quantifying corticosteroid inhibition of adrenocorticotropin release, and they contradict the opposing view, that the release of adrenocorticotropin following noxious stimuli occurs independently of negative feedback control of plasma corticosteroid concentration.

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[3] Present address: Department of Physiology, Stanford University, California.

[4] Postdoctoral Fellow, Neurochemistry Training Grant #2B5241 from National Institutes of Health, Public Health Service, Bethesda.

[5] Work done during tenure as Post Doctoral Fellow of the National Institute of Arthritis and Metabolic Diseases.

THE measured concentration of adrenal cortical hormones in blood is affected only slightly, if at all, by wide variations in the rate of corticosteriod inactivation (1–3). This stability of plasma corticosteroid concentration depends upon the matching of adrenal cortical secretion rate to variable rates of corticosteroid inactivation (2) and strengthens the hypothesis that the concentration of corticosteroids in blood is under negative feedback control (4, 5).

A negative feedback controller can operate under either of two conditions: a) at constant setpoint, it stabilizes the variable under control at a value close to that indicated by the setpoint of the controller, despite random or systematic disturbances tending to cause deviation of the controlled variable from the setpoint value; or b) if the setpoint itself is variable, the controller can force the controlled variable to follow closely the changing setpoint. In the performance under varying setpoint, a controller at any moment will be operating to bring the controlled variable toward, and to stabilize it at the instantaneous setpoint value (6).

The experiments to be described were designed to test the hypothesis that the plasma concentration of adrenal cortical hormones is regulated by means of a negative feedback controller which performs at variable setpoint. According to this hypothesis, reset of the controller could account for the elevations in plasma corticosteroid concentration observed following noxious stimuli, as well as for the diurnal periodicity of plasma corticosteroid concentration. If this view is correct, then a dose of corticosteroid sufficient to produce an increment in plasma corticosteroid concentration equal to the increment produced by a noxious stimulus, should prevent entirely the secretion of endogenous corticocosteroids, if it is given immediately before the noxious stimulus. The exogenous steroid would raise the plasma corticosteroid concentration to the new level at which the noxious stimulus resets the controller: no discrepancy between the existing concentration and the new high setpoint following the noxious stimulus would then be detected by the controller. In the absence of a discrepancy between the existing value of the steroid concentration and the setpoint value, the negative feedback controller could not initiate an increase in endogenous corticosteroid release.

In contrast, if a noxious stimulus provokes a release of adrenocorticotropin (ACTH) in a manner independent of feedback control of plasma corticosteroid concentration, then the increments produced by the exogenous corticosteroid and by the noxious stimulus should summate partially or completely.

METHODS

Male albino rats (Holtzman Rat Co., Madison, Wisconsin) weighing between 80 and 150 grams were used in all experiments. Purina laboratory chow and tap water were available *ad lib.* All arterial blood samples to be analyzed for corticosterone content were obtained between 8:00 and 9:30 A.M. The corticosterone analyses were performed in duplicate by a sulfuric acid fluorescence method (7). The raw data were corrected for the

reagent blank only, and not for an assumed biological blank based on adrenalectomized or hypophysectomized animals.

The rats were anesthetized with 5.0 mg./100 gm. B.W. of pentobarbital intraperitoneally. Ten minutes later one of the following experimental variables was introduced, either alone or after an intravenous dose of corticosterone (5–35 μg/100 gm. B.W.):

0.9% saline, intravenously (control group)
ACTH (U.S.P. bovine corticotropin standard), intravenously (0.125 or 0.250 mU/100 gm. B.W.)
histamine (as the phosphate or dihydrochloride), intravenously (100 μg/100 gm. B.W.) laparotomy

After 15 minutes, blood was obtained from the abdominal aorta or by decapitation, and collection was completed within 30 seconds.

When corticosterone was to be combined with one of the other stimuli, it was administered 15–30 seconds in advance of the second stimulus, into a separate vein. All intravenous injections were given in a total volume of 0.2 ml./100 gm. B.W. in saline, into a lateral caudal vein. The corticosterone solutions contained a trace of ethanol and the ACTH solutions were adjusted to pH 2 with HCl. The control saline injections were similarly modified, when appropriate. The doses of ACTH, corticosterone, and histamine were chosen so that the increments in plasma corticosterone concentration observed after 15 minutes would in all cases be less than the maximal increment produced spontaneously in unanesthetized rats 15 minutes after restraint. Thus even the highest plasma corticosterone concentrations observed in these experiments were in the physiological range of corticosterone concentration in our animals.

All doses presented refer to the amounts of the substances given per 100 gm. body weight.

The term "adrenal cortical system" is used to designate all the components of the central nervous system, pituitary, and adrenal cortex which affect the secretion of corticosterone. The letter B refers to corticosterone (Kendall's Compound B). Data presented as means are accompanied by the standard errors of the means.

RESULTS AND DISCUSSION

Part I: *Details of the experimental design*

The data presented in Figures 1–7 are given as increments in plasma corticosterone concentration produced by the experimental variables. The magnitudes of these increments are dependent upon: a) the establishment of a control plasma corticosterone concentration; b) the effect of the anesthetic on the responsiveness of the adrenal cortical system; c) the relation between the amount of corticosterone added during the 15 minute period, and the distribution of the hormone; d) the extent of metabolism of corticosterone during the 15 minute period between the introduction of the experimental variable and collection of the blood sample; e) the nature of the experimental variable itself.

Establishment of the control plasma corticosterone concentration

Table 1 shows the elevations in plasma corticosterone concentration produced by the handling of the animals. Rats taken by surprise and killed within ten seconds had plasma corticosterone concentrations of 7.3 ± 0.71 μg/100 ml. Intraperitoneal injections of pentobarbital caused an increase

TABLE 1. EFFECT OF ANESTHESIA AND A CONTROL INTRAVENOUS INJECTION ON PLASMA CORTICOSTERONE CONCENTRATION

Group	No.	Plasma corticosterone concentration μg/100 ml.
Unanesthetized; blood obtained by decapitation	8	7.3 ± 0.71
Pentobarbital 5 mg./100 gm. B.W. I.P.; blood obtained by decapitation	7	11.1 ± 1.27
Pentobarbital 5 mg./100 gm. B.W. I.P.; blood obtained from abdominal aorta	18	11.4 ± 0.48
Pentobarbital 5 mg./100 gm. B.W. I.P.; 0.2 ml. saline I.V.; blood obtained from abdominal aorta	81 (selected—see Fig. 1)	15.0 ± 0.62
	100 (unselected—see Fig. 1)	18.0

in plasma corticosterone concentration to 11.1 ± 1.27 μg/100 ml. ten minutes later. In these anesthetized animals the corticosterone concentration was the same in aortic blood as in blood obtained by decapitation, and in all subsequent experiments aortic blood was used. The addition of an intravenous injection of 0.2 ml. of 0.9% saline ten minutes after the anesthetic caused a further elevation of plasma corticosterone concentration to 15.0 ± 0.62 μg/100 ml. fifteen minutes after the saline injection. This further increment results from the saline injection, and not from the extension of the time period between the pentobarbital injection and blood collection. From these data, it can be seen that the pentobarbital-anesthetized, saline-injected animals, which constitute the control group used to define increments in plasma corticosterone concentration in all subsequent experiments, had already undergone a response of the adrenal cortical system sufficient to double the plasma corticosterone concentration, compared to the levels measured in unanesthetized, uninjected animals.

The frequency distribution of plasma corticosterone concentrations in 100 control rats studied throughout the course of all the experiments is shown in Figure 1. The distribution is skewed, and we have arbitrarily

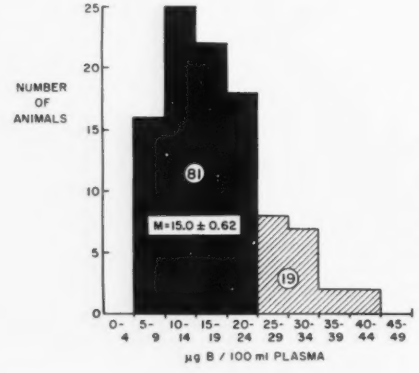

FIG. 1. Frequency distribution of plasma corticosterone concentration in anesthetized, saline-injected control animals. The skewed distribution has been arbitrarily divided into a normal (Gaussian) population of 81 animals, and a population of 19 animals which are presumed to have had a strong adrenal cortical response to the handling associated with administration of the anesthetic.

divided it into two populations. One population of 81 animals showed a relatively normal (Gaussian) distribution of corticosterone concentrations between 4 and 24 μg/100 ml., with a mean of 15.0 ± 0.62. A second population of 19 animals had corticosterone concentrations ranging from 25 to 44 μg/100 ml. We believe these data indicate that in handling our control animals we provoked a strong adrenal cortical response in 20% of the animals. These animals have been eliminated from the control population, and the mean corticosterone concentration of 15 μg/100 ml. has been adopted as the control value, instead of 18 μg/100 ml., which is the mean of the unselected population of 100 animals. The alternative procedure, of accepting the higher mean, and mathematically correcting the skewed population to obtain a standard error, as done by Bliss *et al.* (8), in this case conceals what we believe to be a true heterogeneity of the control group, and so was not employed. The following calculation is given to justify this procedure.

Calculation of increments

The raw data showing the plasma concentrations of corticosterone 15 minutes after introduction of the experimental variables are presented in Table 2. In contrast to the control distribution, the experimental distribu-

TABLE 2. PLASMA CORTICOSTERONE CONCENTRATION IN ANESTHETIZED RATS FIFTEEN MINUTES AFTER INTRODUCTION OF THE EXPERIMENTAL VARIABLES

Group	No.	Plasma corticosterone concentration μg/100 ml.
Corticosterone, I.V.		
5 μg	15	24.5 ± 2.02
7.5 μg	21	29.0 ± 1.37
12.0 μg	30	32.8 ± 1.15
15.0 μg	11	34.5 ± 1.49
30.0 μg	13	49.5 ± 1.94
35.0 μg	8	54.2 ± 1.73
ACTH, I.V.		
0.125 mu	8	23.0 ± 2.70
0.250 mu	10	34.1 ± 1.56
Histamine, I.V.		
100 μg	26	34.9 ± 1.12
Laparotomy	25	40.4 ± 1.37
Corticosterone + ACTH		
5 μg + 0.125 mu.	27	33.4 ± 1.75
12 μg + 0.250 mu.	15	51.3 ± 2.00
Corticosterone + histamine		
12 μg + 100 μg	12	35.9 ± 2.20
Corticosterone + laparotomy		
5 μg + laparotomy	7	40.3 ± 1.32
12 μg + laparotomy	24	38.6 ± 1.28
35 μg + laparotomy	8	56.7 ± 2.29

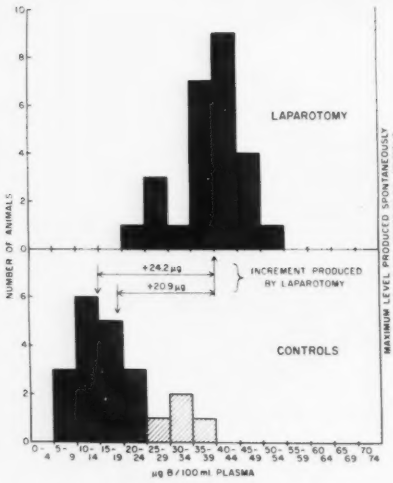

FIG. 2. Frequency distribution of plasma corticosterone concentration fifteen minutes after laparotomy, and in concurrently assayed controls. The arrow on the right indicates the mean of the laparotomy group; the arrow on the extreme left indicates the mean of the control population after elimination of the four values above 24 μg/100 ml.; the remaining arrow indicates the mean of the entire control population, without selection.

tions did not appear to be skewed (e.g. laparotomy in Fig. 2), and in these no selection was attempted.

The various increments in plasma corticosterone concentration produced by the experimental variables were calculated by subtracting the mean concentration of the selected control population from the mean concentration of each unselected experimental population shown in Table 2. The standard error of the increments was expressed by:

S.E. of increment

= S.E. of the difference between the means of two Gaussian distributions

$$= \sqrt{(\text{S.E.}_{M1})^2 + (\text{S.E.}_{M2})^2}$$

A representative example of the slight effect of the selection of the control population on the magnitude of an increment appears in Figure 2. This figure shows all the raw data obtained from 26 rats following laparotomy, and from 21 control animals sampled concurrently. Even in this small group of controls the skewed distribution is evident. Without selection of the control population, the calculated increment following laparotomy is 20.9 μg/100 ml. After correction of the control population by elimination of the four values above 24 μg/100 ml., the calculated increment following laparotomy becomes 24.2 μg/100 ml. The interrelations among the increments shown in Part II, and the statistical significance of the differences discussed, are not appreciably altered by the selection of the control group.

Effect of the anesthetic on the response of the adrenal cortical system to an ACTH-releasing stimulus

Pentobarbital depresses the hypothalamus (9, 10), and since hypothalamic structures may be involved in the response of the adrenal cortical

system to a stimulus such as histamine (11), we have compared the increment in plasma corticosterone concentration produced by histamine in unanesthetized and in anesthetized rats. In 11 unanesthetized rats, brief restraint plus the intravenous injection of saline produced an increment in plasma corticosterone concentration of 13.4 ± 1.8 μg/100 ml., and brief restraint plus intravenous injection of 100 μg histamine in 14 rats produced an increment of 25.8 ± 2.8 μg/100 ml. Unanesthetized rats shown in the first line of Table 1 were the controls.

The levels in rats under pentobarbital were higher than in the unanesthetized animals, and saline injection increased them by 4 μg/100 ml., as indicated in Table 1. Nevertheless, the injection of 100 μg of histamine in 26 anesthetized animals produced an increment of 19.9 ± 1.12 μg/100 ml. above the levels in the anesthetized, saline-injected control group. Thus, the response of the adrenal cortical system to intravenous histamine does not appear to be depressed by pentobarbital in the dose used in these experiments.

Relation between dose of corticosterone and increment in plasma corticosterone concentration

The virtual volume of distribution of corticosteroids is dependent upon the dose of the steroid administered: tracer doses have smaller distribution volumes than do pharmacological doses (2, 12). We considered it possible that the various physiological doses used in our experiments might have had different volumes of distribution, which would affect the increments in plasma corticosterone concentration produced by the steroid. Therefore, the relationship between the increment in plasma corticosterone concentration at 15 minutes and the amount of corticosterone added at zero time was examined. The results are shown in Figure 3. The relationship is nonlinear, but from these data it appears that increments in plasma corticosterone concentration larger than 5 μg/100 ml. 15 minutes following an experimental stimulus are linearly related to the amount of corticosterone added to the extra-adrenal pool of the steroid.

Effects of corticosterone metabolism on increments in plasma corticosterone concentration

Variations in the rate of hepatic inactivation of corticosterone may produce opposite variations in the increment in plasma corticosterone

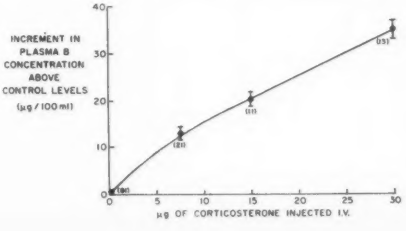

FIG. 3. Relationship between the amount of corticosterone injected intravenously, and the increment in plasma corticosterone concentration fifteen minutes later.

concentration observed 15 minutes after a given intravenous dose of this hormone (13). From previous studies of the effects of ACTH and adrenal cortical hormones on hepatic inactivation of corticosterone (5), we have concluded that no detectable variations in the activity of the hepatic enzymes which inactivate corticosterone would be caused by either ACTH or corticosterone under the conditions of the present experiments. Since the increment in plasma corticosterone concentration produced by corticosterone in the presence of histamine was the same as the increment produced by corticosterone alone (Fig. 5), it can be concluded that histamine did not impair the metabolism of the steroid: if histamine had slowed corticosterone metabolism, less of the steroid would have disappeared in the 15 minutes following the injection, and the amount remaining (the increment) would have been greater than that observed after the corticosterone alone.

We expected that laparotomy might cause a transient diminution in the rate of steroid metabolism through impairment of hepatic blood flow following splanchnic vasoconstriction associated with opening the abdomen. However, the increment produced by 35 μg of corticosterone was the same in the presence of laparotomy as it was after the corticosterone alone (Fig. 6), and so we concluded that laparotomy also did not significantly impair corticosterone metabolism.

Summation of increments in plasma corticosterone concentration produced by ACTH and by corticosterone

Recently it has been suggested that exogenous corticosterone inhibits the synthesis of endogenous corticosterone following ACTH by an action on the adrenal (14). However, in the experiments in Part II the assumption is made that exogenous corticosterone in the doses and conditions employed does *not* interfere with release of endogenous corticosterone from the adrenal cortex in response to ACTH. The data shown in Figure 4 demon-

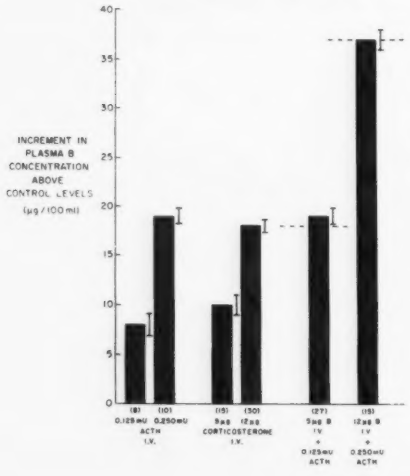

FIG. 4. Summation of the increments in plasma corticosterone concentration produced by corticosterone and by ACTH, at two dose levels. The exogenous corticosterone does not impair the release of endogenous corticosterone following ACTH, at these dose levels. The horizontal dashed lines indicate the sums of the separate corticosterone and ACTH increments shown in the left and middle pairs of columns.

strate that this assumption is valid: at both dose levels tested, the summation of the ACTH-produced increment and the exogenous corticosterone-produced increment is exact. It follows from these results that in any experiment involving the conbination of a corticosterone injection and administration of an adrenocorticotropin-releasing stimulus, the increment in plasma corticosterone concentration attributable to endogenous ACTH release will be equal to the total increment observed, minus the increment observed with the corticosterone alone.

Part II: *Evidence for the resetting of the negative feedback control of plasma corticosteroid concentration*

Histamine study

According to the hypothesis that certain stimuli provoke increases in plasma corticosteroid concentration by resetting the negative feedback control of the concentration, a dose of corticosterone sufficient to produce an increment in plasma corticosterone concentration equal to that produced by the stimulus itself, should prevent entirely the release of ACTH following that stimulus. Figure 5 shows the results obtained with histamine

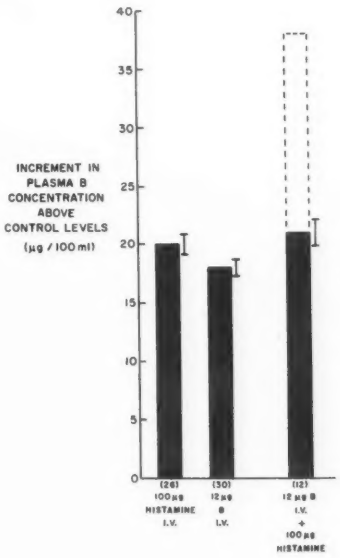

FIG. 5. Increments in plasma corticosterone concentration produced by histamine and corticosterone given separately, and together, 15 seconds apart. The dashed column indicates the sum of the separate increments, shown in the left and middle columns. The increment, when the corticosterone injection is given 15 seconds prior to the histamine injection, is not significantly different from that observed with the corticosterone alone.

as the stimulus to produce ACTH release. Injections of histamine and corticosterone, matched to produce equal increments in plasma corticosterone concentration, were given in the same animal. Only the increment produced by the exogenous corticosterone was then observed. The ACTH release ordinarily provoked by histamine was entirely prevented by this dose of corticosterone. Had there been any ACTH release, partial or

complete summation of the increments would have been observed, as was the case when ACTH and corticosterone were given together (Fig. 4).

Laparotomy studies

The reset hypothesis also predicts that the increased secretion of corticosterone after a noxious stimulus will always be of exactly the magnitude necessary to bring the plasma corticosteroid concentration to the new level set by that stimulus. To test this prediction we first determined the increment produced by laparotomy. We selected two doses of corticosterone (5 and 12 μg) which produced increments less than that observed after laparotomy, and one dose (35 μg) which produced an increment greater than that after laparotomy. Laparotomy was then combined with each of these doses of corticosterone. The results are shown in Figure 6.

When laparotomy was combined with either the 5 or 12 μg dose of corticosterone, the total increment observed was the same as after laparotomy alone. Thus, the increment in plasma corticosterone concentration produced by endogenous ACTH release was dependent on the size of the increment produced by the exogenous corticosterone: in each case it was diminished exactly to the degree necessary to bring the plasma corticosterone concentration to the level set by the stimulus of laparotomy alone.

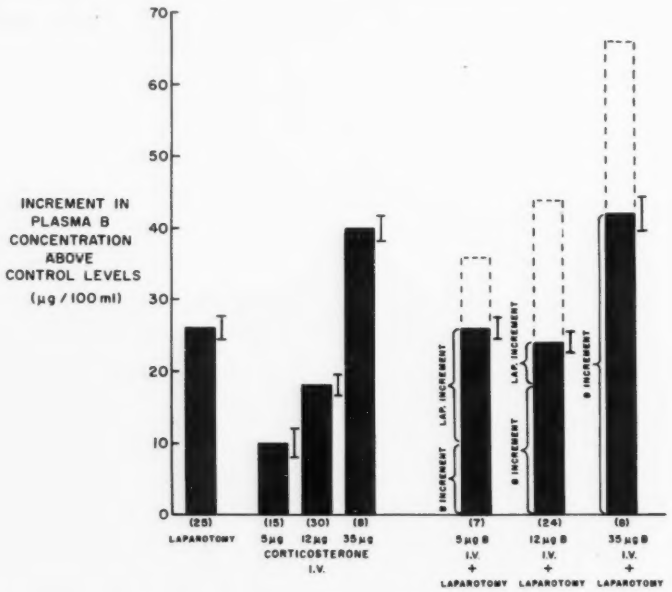

FIG. 6. Increments in plasma corticosterone concentration following laparotomy, and various doses of corticosterone. The dashed columns indicate the sums of the separate laparotomy and corticosterone increments, which are shown in the left half of the figure.

Had laparotomy provided a direct drive of adrenocorticotropin release, independent of plasma corticosteroid concentration, complete summation of the increments (indicated by the dashed lines in the figure) would have been observed, as shown by the data in Figure 4.

The 35 μg dose of corticosterone provided an increment in plasma corticosterone concentration higher than that observed after laparotomy. According to the reset hypothesis, no ACTH release could occur if laparotomy were combined with such a dose of exogenous corticosterone, since the exogenous corticosterone would bring the plasma corticosterone concentration to a value higher than the new level set by laparotomy. As shown in Figure 6, the increment observed in this case was exactly that of the 35 μg of corticosterone alone, and no release of ACTH occurred.

In the experiments described so far, measurements of the increments were made 15 minutes after the introduction of the experimental variables. In order to ascertain whether our results were critically dependent upon this arbitrary choice of timing, we extended the studies of the increment produced by 12 μg corticosterone and by laparotomy, separately and together, from 3 to 40 minutes. The results are shown in Figure 7.

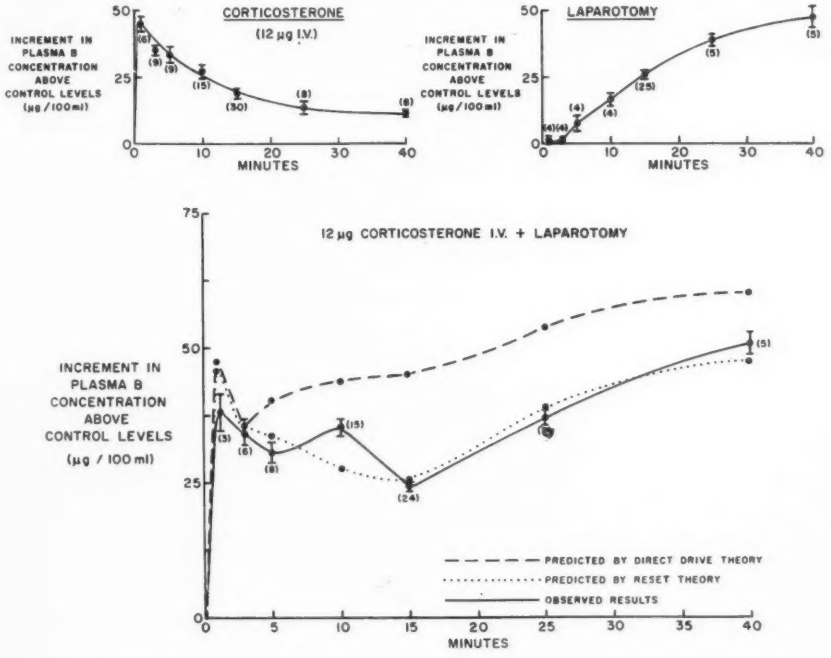

FIG. 7. Time course of the increments in plasma corticosterone concentration following 12 μg/100 gm. B.W. of corticosterone intravenously, or laparotomy. The derivations of the two theoretical curves, indicated by the dotted and dashed lines, are given in the text.

The increment produced by 12 μg corticosterone decays in a roughly exponential manner, as would be expected from the first-order kinetics of corticosterone metabolism in intact rats (15, 16). Following laparotomy, the plasma corticosterone concentration does not begin to increase until after 3 minutes, and then there is a progressive increase throughout the remainder of the period of observation.

According to the reset hypothesis, the combination of laparotomy and 12 μg corticosterone would give: a) an increment equal to that produced by the corticosterone alone for the time period during which the exogenous corticosterone increment exceeds or equals that produced by laparotomy alone (see 35 μg corticosterone plus laparotomy in Fig. 6, and histamine plus corticosterone, Fig. 5); and b) an increment equal to that produced by laparotomy alone for the time period during which the laparotomy increment exceeds that produced by exogenous corticosterone alone (see 5 or 12 μg corticosterone plus laparotomy—Fig. 6). These predictions of the reset hypothesis are shown by the dotted line in Figure 7.

In contrast, if laparotomy provides a direct drive for ACTH release, independent of the negative feedback control of plasma corticosteroid concentration, then at all points in time the laparotomy and exogenous corticosterone increments should summate exactly (as in the case of ACTH plus corticosterone—Fig. 4). This prediction of the direct-drive hypothesis is shown by the dashed line in Figure 7.

The experimental results are shown by the solid line in Figure 7. The experimental curve followed closely that predicted by the reset hypothesis, except at the 10 minute point. The deviation at 10 minutes was confirmed on several occasions. It could not be accounted for by an impairment of corticosterone metabolism following laparotomy, because the 10 minute increment produced by a test dose of 35 μg of corticosterone in six animals was not significantly different in the presence of laparotomy (54 ± 4.8 μg/100 ml.) from the 10 minute increment produced in six animals by the 35 μg dose of corticosterone alone (47 ± 3.5 μg/100 ml.). In the previous experiments, it appeared that the levels 15 minutes after histamine or laparotomy must have been at or very close to the instantaneous setpoint value: there was no summation of the exogenous corticosterone plus laparotomy or histamine increments beyond the levels produced by histamine or laparotomy alone. However, if the setpoint increases five to ten minutes after laparotomy more rapidly than the levels can follow, then the levels at 10 minutes on the laparotomy curve (upper right, Fig. 7) would be below the instantaneous setpoint value, instead of close to it. In such a case, increments produced at 10 minutes by small doses (e.g. 12 μg) of exogenous corticosterone would summate with the laparotomy increment to the extent necessary to bring the concentration to the setpoint value. By the fifteen minute point and afterward, the levels presumably are close to the setpoint, and change with it *pari passu* at a rate slower than the initial rate

of change of the setpoint, which was too rapid for the plasma corticosterone concentration to follow closely.

The results contradict the direct-drive hypothesis at every point at which the two hypotheses predict different results.

The studies described in this paper demonstrate that corticosterone in physiological concentrations may act as a potent inhibitor of adrenocorticotropin release, and that the degree of inhibition is dependent upon both the strength of the ACTH-releasing stimulus and the dose of corticosterone administered. But further, they show that the increments in plasma corticosterone concentration produced by stimuli such as histamine or laparotomy are dependent in a predictable and formal manner upon the pre-existing corticosterone concentration. We interpret the evidence that plasma corticosterone concentration is increased at a given time after a given stimulus by a particular amount, and that ACTH release occurs only to the degree necessary to achieve that particular corticosterone concentration, to mean that the negative feedback control of plasma corticosteroid concentration is reset by the stimuli studied to regulate at new, higher setpoints.

In 1947 the now well-known studies of Sayers and Sayers first demonstrated the quantitative relationship between such stimuli as cold or histamine and ACTH release, as estimated by adrenal ascorbic acid depletion (17). They also showed the quantitative relationship between the dose of adrenal cortical extract or crystalline corticosteroids used as pre-treatment, and the resulting inhibition of ACTH release following the same stimuli. Two conclusions were: "The degree of inhibition of pituitary adrenocorticotrophic activity is proportional to the amount of administered cortical hormone" and "The greater the degree of stress to which the animal is subjected, the greater is the amount of cortical hormone required to inhibit pituitary adrenocorticotrophic activity." The present studies, taking advantage of a recent method for direct estimation of plasma corticosteroid concentration, confirm both of these conclusions.

Sayers and Sayers supposed that "the great variety of non-specific stresses increase pituitary adrenocorticotrophic activity by a common mechanism, namely by increasing the requirement of the peripheral tissue cells for cortical hormone(s). The anterior pituitary responds to the decrease in concentration of cortical hormones in the body fluids by increasing the rate of elaboration of A.C.T.H." The subsequent discoveries, after suitable methods became available, that the same stimuli which cause adrenal ascorbic acid depletion also cause increases in plasma corticosteriod concentration and that they may also prolong the biological half-life of corticosteroids (13), discredited this supposition, and seemed to indicate that a negative-feedback hypothesis could not explain the adrenal cortical response to a great variety of stimuli.

The present studies represent an attempt to show that the rises in plasma

corticosteroid concentration following varied stimuli are in fact consistent with the view that the plasma concentration of corticosterone in the rat (and presumably of cortisol in many other species) is at all times under effective negative feedback control (providing no ablations or lesions have been made). The increase of the setpoint of the controller creates a *virtual* drop in plasma corticosteroid concentration which then provides a signal indistinguishable to the controller from an absolute drop in concentration such as required by the original view of Sayers and Sayers. Such a resetting of a negative feedback controller is commonplace in automatic control engineering, and is familair in physiology as one explanation for regulation of elevated body temperature during fever.

Acknowledgments

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ELECTRON MICROSCOPIC STUDIES OF ANTERIOR PITUITARY GLANDS FROM LACTATING AND ESTROGEN-TREATED RATS[1]

W. C. HYMER, W. H. McSHAN AND R. G. CHRISTIANSEN

Department of Zoology, University of Wisconsin, Madison, Wisconsin

ABSTRACT

Anterior pituitary glands from mature virgin female, estrogen, treated, and lactating rats were studied with the electron microscope. It was found that the endoplasmic reticulum underwent extensive change during lactation. A similar situation was also found in the pituitaries of the estrogen treated animals. This well developed reticulum was localized mainly in the acidophils with 600 mμ granules, indicating these cells are probably responsible for the elaboration of lactogenic hormone. In addition to thin sections for electron microscopy, thick sections were taken for light microscopy. The pituitaries from the animals which were injected with estrogen were assayed for lactogenic hormone. On the basis of these results and those of others a scheme is presented for production and release of lactogenic hormone from certain of the acidophilic cells.

SEVERAL workers have reported significant changes in the acidophils of the rat pituitary during lactation. These changes, as seen with the light microscope, are characterized by a degranulation of a high proportion of acidophils with concomitant enlargement of the Golgi region and enlargement of basophilic inclusions in the cytoplasm (4, 5, 6). Everett and Baker (6) reported a post partum increase in the cells which stain with acid dyes accompanied by an increase in the lactogenic hormone content of the pituitary after parturition.

The effect of estrogens on pituitary cytology has been investigated with the light microscope. Severinghaus (22) found that an activation of the acidophils was induced by the administration of large amounts of estrogen, whereas Baker and Everett (1) and Meyer *et al.* (15) showed that small doses of estrogen could also exert a stimulative action on the acidophils. Foster (10) found an increase in the amount of vacuolation and strongly sudanophilic material (Golgi elements) accompanied by a coalescence of vacuoles when estrogen was administered to rats. Further evidence for the effect of estrogen on the acidophils was given by Purves and Griesbach (18) in a study in which two types of acidophils in the rat pituitary were differentiated by their responses to experimental treatment. When rats were treated with estrogen, they found a degranulation of those acidophils

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located on the interior of cell cords. They suggested this type of acidophil was lactogenic in function.

Farquhar *et al.* (7, 8, 9, 11, 20) studied the rat anterior pituitary gland with the electron microscope. She has reported "parallel arrays of cisternae" and "delicate canaliculi (or lamellae)" associated with the normal acidophilic cells of the rat anterior pituitary. These structures change quite dynamically; associated with canalicular increases are corresponding decreases in granulation. This would indicate that these structures develop in relation to a changing functional state.

Since it is known that large doses of estrogen can induce high levels of lactogen in the pituitary (14), and that pituitary lactogenic hormone content is high during lactation (12), it was of interest to determine whether correlations between the two situations could be detected at the submicroscopic level.

MATERIALS AND METHODS

Observations were made on pituitaries taken from non-pregnant, non-lactating females and lactating female rats of the Holtzman strain. The following series of animals, in addition to the non-pregnant, non-lactating controls, were used in this part of the study: at parturition, 2, 7, 10, 14, 22, 25 and 32-day lactating animals (young not weaned); 1 month post-lactating (young weaned at 21 days).

Mature (200–250 gm.) virgin female rats obtained from the Holtzman Company, Madison, Wisconsin, were used for the estrogen experiments. Total doses of 50, 100, and 400 μg of estrone were injected subcutaneously into one rat each for 5 days. This experiment was repeated once. The pituitaries were taken on the 6th day and fixed for electron microscopy.

The tissue was fixed with buffered Palade fixative (pH 7.4) for 2 hours, stained in a 0.5% uranyl acetate alcohol solution for 0.5 hour, rapidly dehydrated in increasing concentrations of methyl alcohol, and finally placed in a n-butyl-methyl methacrylate mixture with 2% benzoyl peroxide used as catalyst, and the tissue was then incubated at 46° C for 24–32 hours. Thin sections were obtained with a Servall ultramicrotome and observed with either a RCA EMU-2 or a Siemens Elmiskop IIb electron microscope.

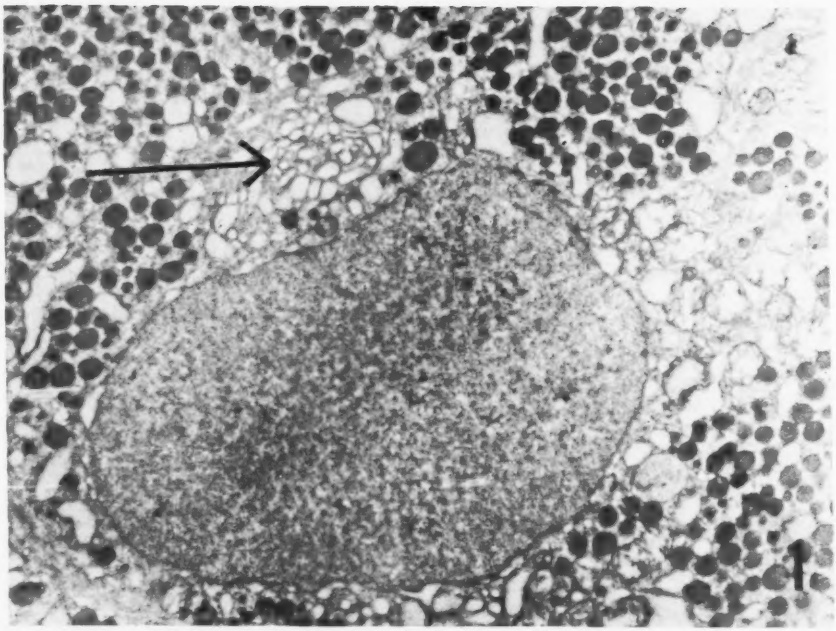
To study similar areas with the light microscope, adjacent thick sections were taken. These sections (4.5–5.5 μ) were stained according to the colloidal iron method of Jennings *et al.* (13).

A series of animals (virgin females) were injected with 50, 100, and 400 μg of estrone and their pituitaries were assayed for lactogenic hormone content by the pigeon crop method of Breitenbach and Meyer (2) using intradermal injections over the crop sac.

RESULTS AND DISCUSSION

Normal and lactating rats

Sections of pituitaries taken from normal female rats are shown in Figures 1 and 2. The important things to be noted in these micrographs are: 1) in Figure 2 the granules can be seen to be bound by a smooth surfaced membrane as originally observed by Farquhar and Wellings (9) and 2) the endoplasmic reticulum in the acidophils with the 350 mμ granules is not as well developed as in the acidophils with the 600 mμ granules. It should be mentioned here that the diameter of these large acidophilic



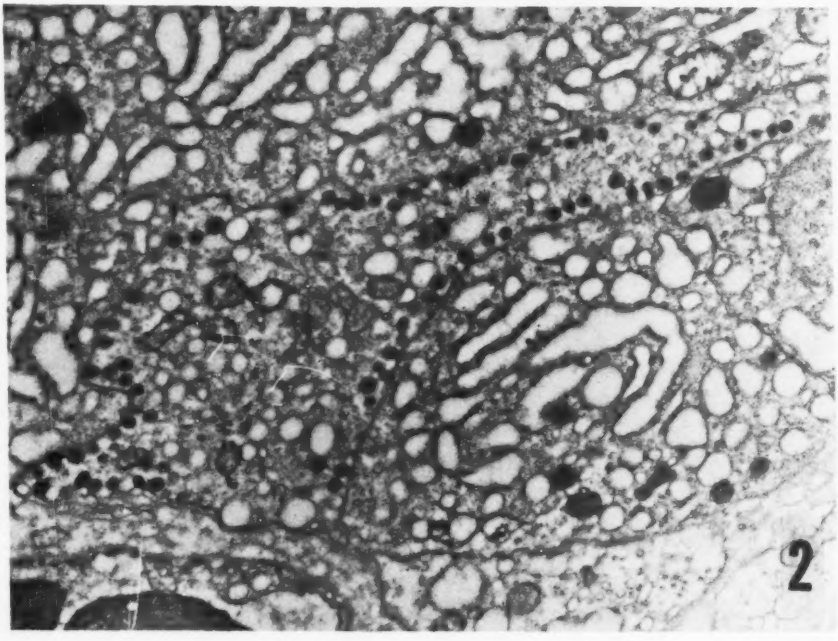


FIG. 1. An acidophil which contains 370 mμ (maximum) granules in the cytoplasm. This cell is probably a growth hormone producing cell. Note the well defined Golgi area (arrow). This tissue was taken from a normal female animal. ×15,000.

FIG. 2. An angular thyrotrope bound by two acidophils. The 150 mμ thyrotropic granules have a well defined membrane around them. Note the large acidophilic granules (500 mμ diameter) closely allied to the extensively developed endoplasmic reticulum (with associated ribosomes). Normal female pituitary. ×19,000.

granules is variable, the range being 400 to 700 mμ. For purposes of clarity, however, these acidophilic granules will be referred to as the 600 mμ granules as originally designated by Farquhar and Rinehart (7).

The results obtained with rat pituitaries during lactation indicate that a high degree of endoplasmic reticulum or canalicular formation is associated with the acidophils during all stages of the 21-day lactation period. It appears that these formations reach their greatest development during 7 to 14 days of lactation. The micrographs in Figures 3 and 4 taken from glands of 14-day lactating animals show extensive development of the endoplasmic reticulum in the acidophils with 600 mμ granules. The appearance of these cells suggests that: 1) they have secreted their hormonal product (lactogenic hormone) because there is an extensive degranulation, and 2) that a rapid rate of protein synthesis is occurring because of the extensive formations of the endoplasmic reticulum.

During the lactation period (as in the non-pregnant, non-lactating animal) basophils, and acidophils containing 350 mμ granules, do not have this well developed endoplasmic reticulum. There is a suggestion that the mitochondria in the acidophils with the 600 mμ granules are somewhat more distended than their counterparts in the basophils, which are long filamentous structures with well defined cristae.

The pituitary cytology of the one month post-lactating animals (young weaned at 21 days) appears to approximate the normal. The endoplasmic reticulum does not show the distentions readily observed in the pituitaries from lactating animals.

Estrogen treated rats

Reece and Turner (19) showed that the lactogenic hormone content of rat pituitary glands was increased on administration of estrogen. The doses of estrogen used in our experiments were those used by Meites and Turner (14). Fifty, 100, and 400 μg of estrone were injected over a 5-day period to mature female rats. It was found that the lower or intermediate doses increased the lactogen content to a greater extent than the larger dose. In no case was the lactogen content reduced below the control level (Table 1). These results confirm those obtained by Meites and Turner (14).

The animals which received 50 μg of estrogen showed two important and readily observable changes from the normal. First, the endoplasmic reticulum was well developed in the acidophils. Frequently one can find the "Nebenkern" formations of Farquahr and Rinehart (7) although these occur with perhaps greater frequency in the pituitaries of animals treated with the higher doses of estrogen (Fig. 10). The endoplasmic reticulum is not well developed in the basophils. Thus, essentially similar formations of the reticulum in the lactating and estrogen treated rat pituitaries would suggest that the acidophilic cells with large granules are intimately associated with the production and release of lactogenic hormone, as was recently suggested by Hedinger and Farquhar (11). Since the pituitary levels of

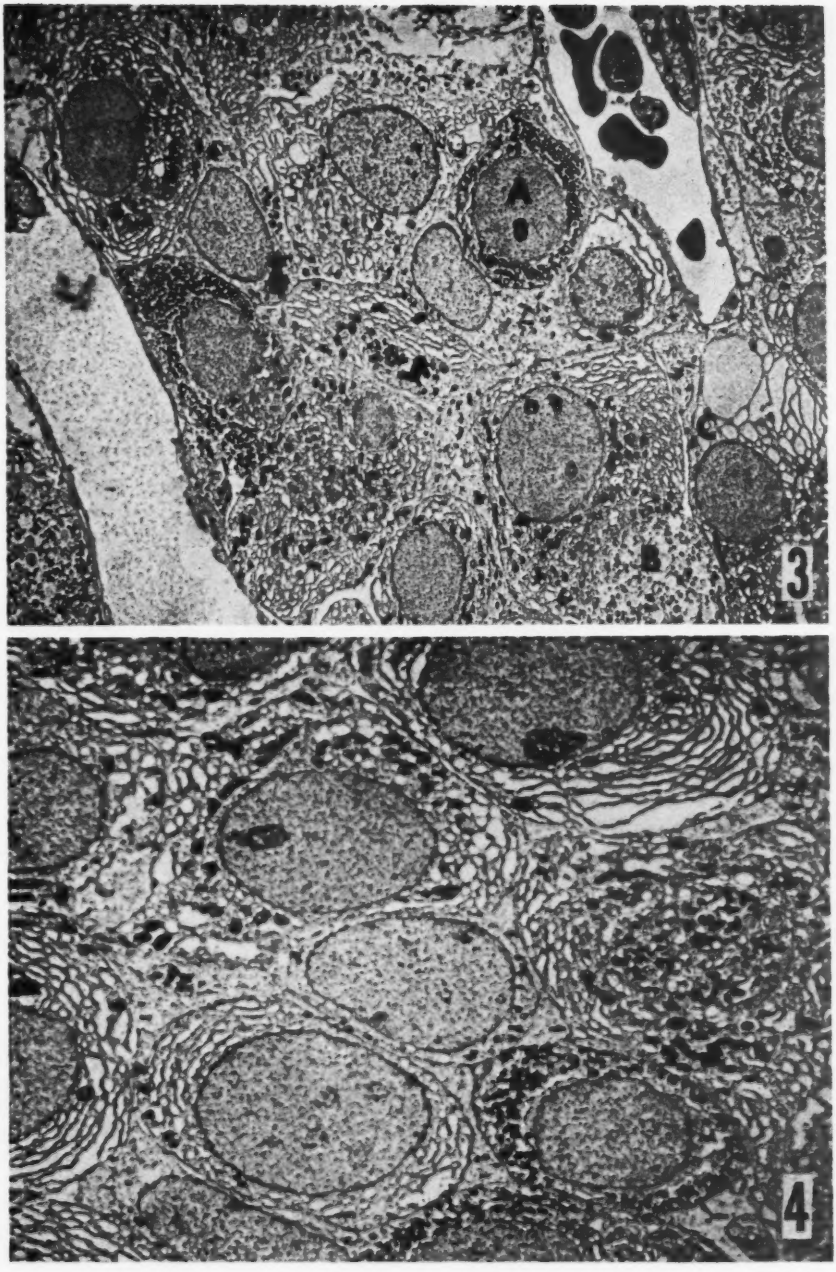


FIG. 3. A low power electron micrograph from a pituitary gland of a 14-day lactating rat. An acidophil (A) is seen lining a sinusoid. Most of the granules in this cell have a diameter of 300 mμ, while a few of the larger are 350 mμ. A few gonadotropic basophils with 200 mμ diameter granules are also present (B). Apparent in the field are parallel arrays of endoplasmic reticulum. In the cytoplasm of these cells are a few large acidophilic granules which measure about 600 mμ (C). ×3500.

FIG. 4. Same tissue as Fig. 3. Note extensively degranulated cells which contain a few large acidophilic granules. ×6500.

TABLE 1. EFFECT OF GRADED DOSES OF ESTRONE ON LACTOGENIC HORMONE LEVELS IN THE RAT PITUITARY GLAND

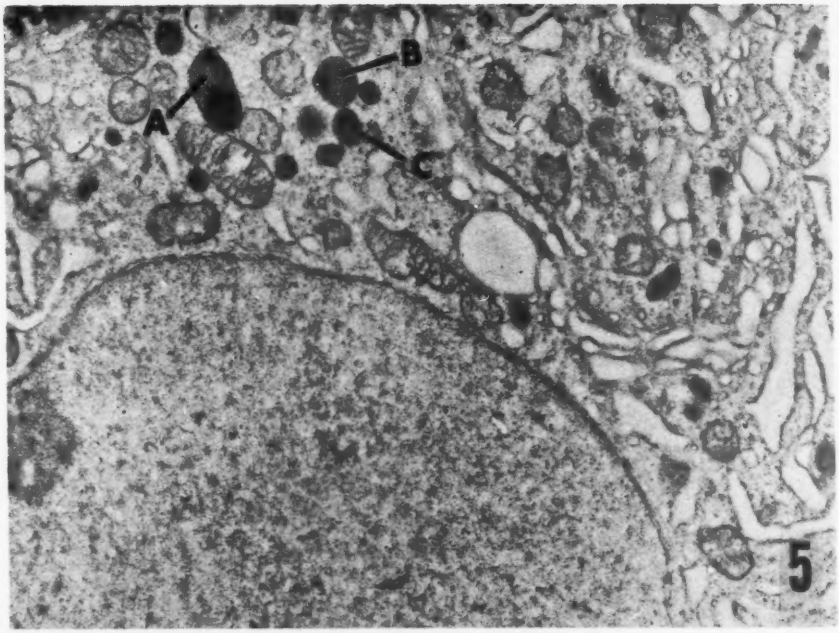
Amount of estrone injected into female rats (μg)	Body wt. (gm.)	Pituitary glands		Response	Crop wt. of stimulated area (mg.)	Crop area (sq. cm.)
		Weight (mg.)	Amount injected into pigeons[1] (mg. fresh tissue)			
Control*			1	Neg.†		
0	246	10.7	2	+	115	3.1
			4	0 +	62	1.3
			.5	++	266	8.2
50	248	8.4	1	++	541	19.7
			2	+	141	4.4
			.5	++	364	9.8
100	252	12.2	1	++	570	13.9
			2	++	409	10.1
			.5	++	321	11.6
400	242	12.8	1	++	299	8.3
			2	+	122	2.5

[1] 6-week-old white king pigeons were used.
* Virgin female rats were used.
† Each reading represents an average of 2 observations on different pigeons.

other tropic hormones were not assayed, the possibility of a change in the level of these hormones cannot be excluded. Secondly, as seen in Figures 5 and 6, "granule within granule" formations appear frequently in the pituitaries of the animals treated with estrone. They have been seen very rarely in normal tissue, and only slightly more often in pituitaries from lactating rats. Recent studies of mouse liver (3) show bodies which appear morphologically similar to those in Figures 5 and 6. In the liver these bodies contain high acid phosphatase activity. Since it has also been shown that acid phosphatase levels are high in the anterior pituitary of the rat after experimental manipulations (21), these bodies found in the pituitary after estrogen administration may be similar to the acid phosphatase bodies in the mouse liver.

The animals which received 100 μg of estrone, the dose which causes high pituitary levels of lactogenic hormone (Table 1), showed no outstanding differences in their pituitary cytology from those which received 50 μg. The cell shown in Figure 7 has probably secreted its hormone (evidenced by the degranulation) and may be in the process of synthesizing new hormone as evidenced by the possible condensation of products in the Golgi region to form granules.

The pituitaries taken from animals receiving 400 μg of estrone possessed the same characteristics although perhaps to a greater extent than those from rats given the smaller amounts (Figures 8–10). It is of interest that one finds more granules being formed in the Golgi region of pituitary glands of estrogen-treated rats than in those taken from non-pregnant, non-lactat-



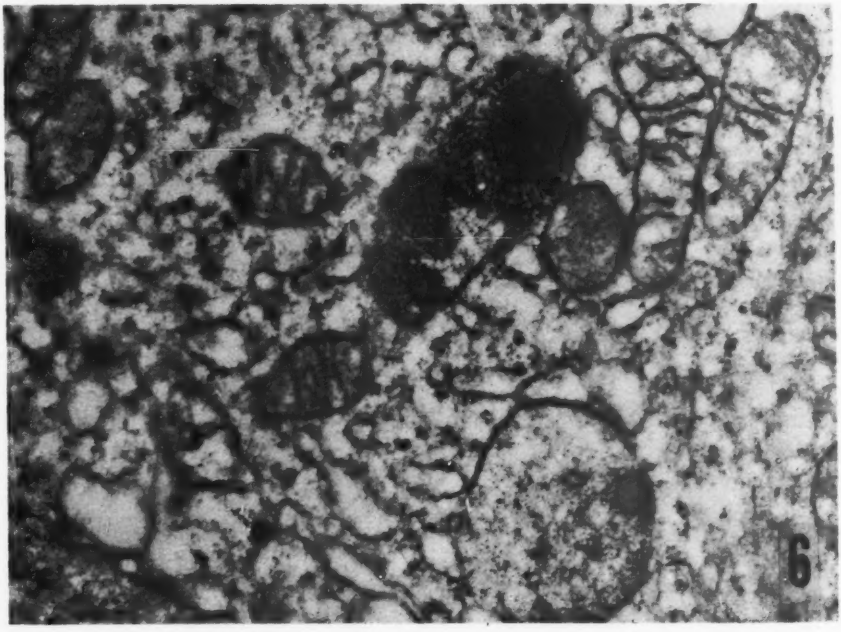


FIG. 5. An acidophil from a pituitary gland of a rat given 50 μg of estrone. The granular structures with more electron dense granules within are of interest. The largest (A) has a maximum diameter of 750 mμ, while the large granule within it measures 400 mμ; structure (B) has a diameter of 450 mμ, while its inner granule measures 200 mμ; and (C) has a diameter of 350 mμ, while its largest inner granule is 200 mμ. Several other smaller granules are also apparent. ×19,000.

FIG. 6. Higher magnification of "granule within granule" structure, from a rat treated with 400 μg of estrone. It measures 1250 mμ maximal diameter with the inner granules measuring 490, 265, and 143 mμ. ×39,000.

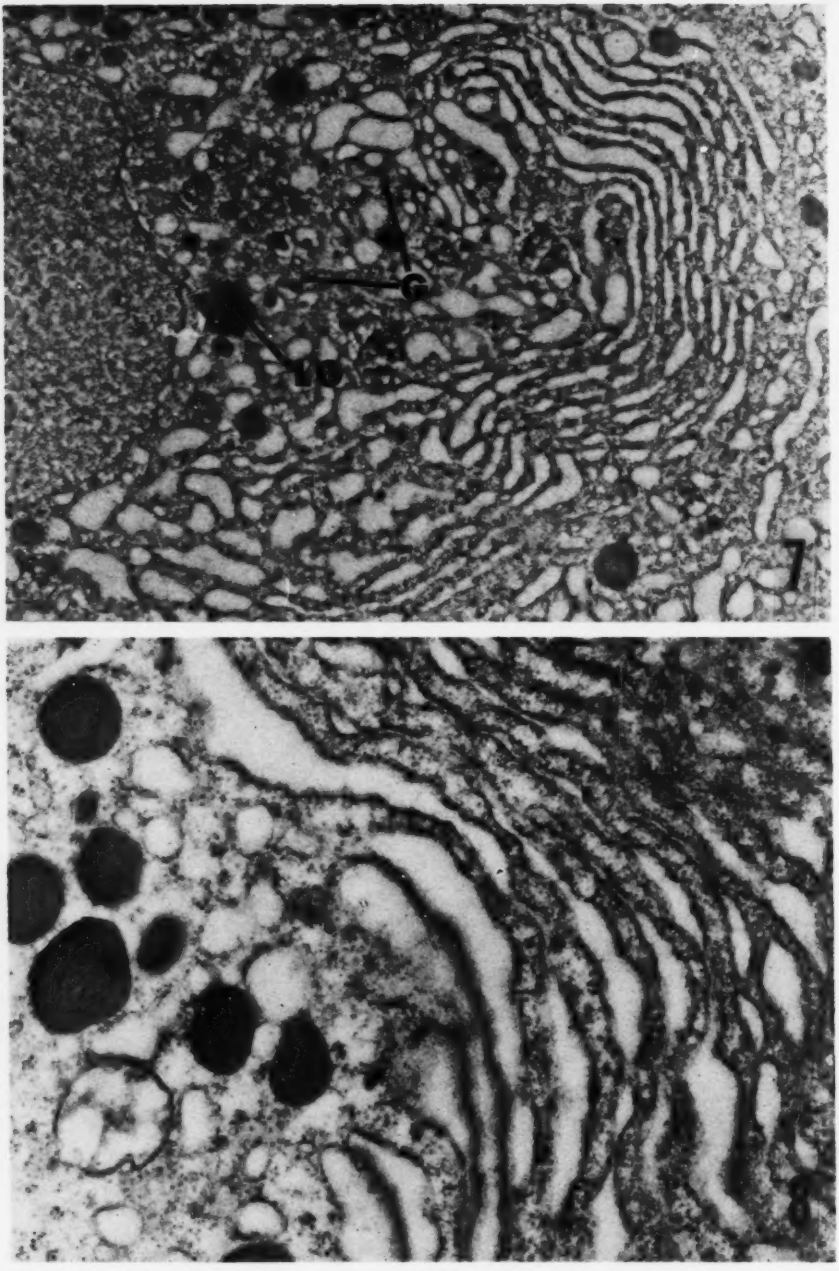


FIG. 7. Section of pituitary gland from a rat treated with 100 μg of estrone. This cell shows the parallel arrays of the endoplasmic reticulum which at some points connect to form vesicles. The Golgi region is seen at (G), and contains granules being formed within its elements. The very large granule (LG) measures about 500 mμ and the granules within the Golgi region range from 40 to 200 mμ. $\times$19,000.

FIG. 8. Section of pituitary gland from a rat treated with 100 μg of estrone. Largest granules measure from 450 to 550 mμ. Attached and free ribosomes are obvious in their associations with the bizarre formations of the endoplasmic reticulum. Apparent structure in granules is sectioning artifact. $\times$39,000.

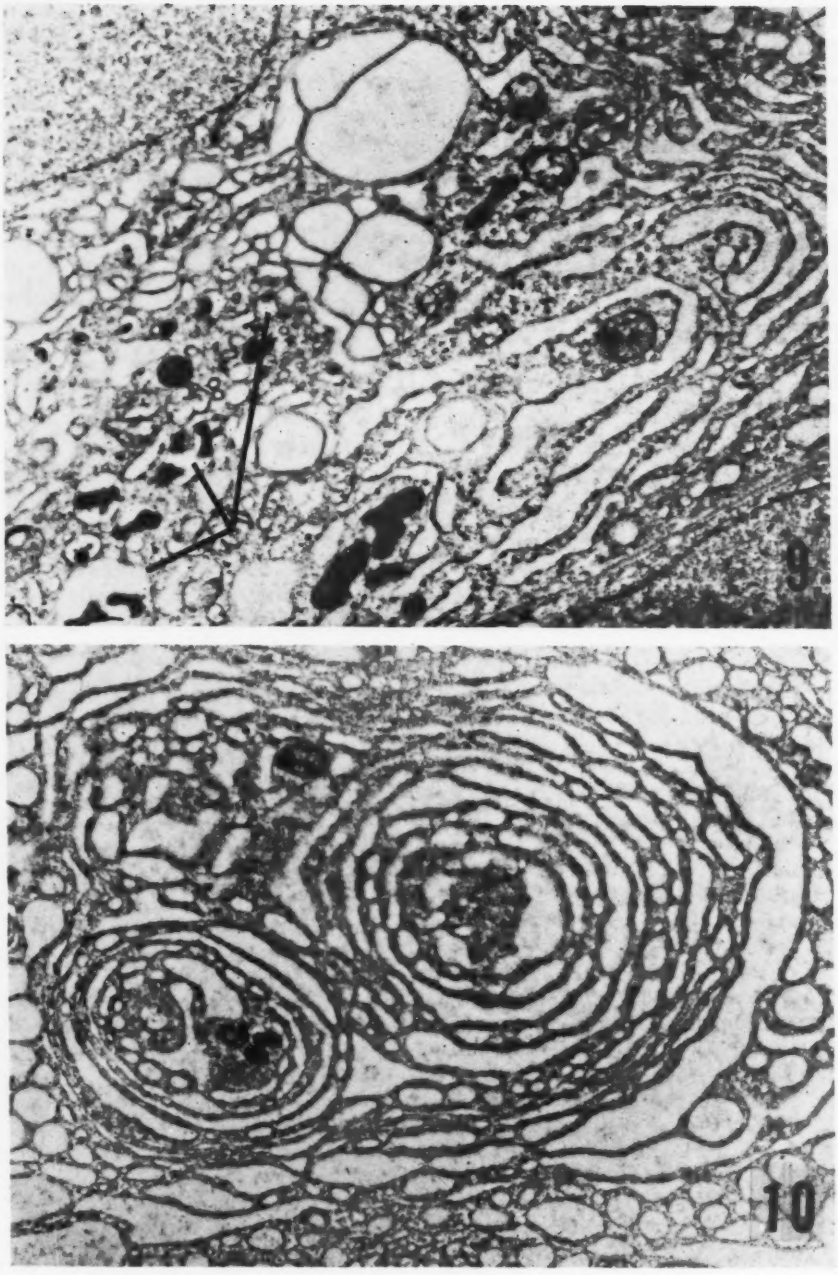


FIG. 9. An acidophil from a pituitary gland of a rat given 400 μg of estrone, similar to that in Fig. 7, showing extensive Golgi development and granule formations within this region. $\times$23,000.

FIG. 10. Electron micrograph of a thin section of a pituitary gland taken from a rat treated with 400 μg of estrone showing a "Nebenkern" formation described in the pituitary by Farquhar. $\times$20,000.

ing rats. This finding is not surprising since estrogen administration presumably results in a rapid synthesis of lactogenic hormone (Table 1).

These results indicate that dynamic changes occur in the endoplasmic reticulum of the acidophils with the 600 mμ granules during estrogen administration. Such changes are very similar to those found in lactating animals. The morphological findings indicate that lactogenic hormone is probably produced, stored and secreted by these cells. The mechanism of production, storage and secretion is probably similar to that reported by other workers, and may proceed according to the following scheme. Hormone synthesis probably occurs within the well developed endoplasmic reticulum area. The fact that the reticulum is studded with ribosomes indicates that rapid protein synthesis is occurring. Rinehart and Farquhar (20) have suggested that elements of the Golgi apparatus may communicate with the endoplasmic reticulum, and Palade (16) suggests that the endoplasmic reticulum may function as an intracytoplasmic transport system. Thus the newly synthesized hormone (not seen in the form of Palade's intracisternal granules (17)) would be carried to the Golgi region where condensation of the hormone would result in granule formation and subsequent envelopment by a smooth membrane (9). The mature granules within their smooth membrane would then move out to be stored in the peripheral cytoplasm until extruded into the perisinusoidal space for secretion into the blood stream.

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PITUITARY CONTROL OF THE TESTIS OF THE HYPOPHYSECTOMIZED RAT[1]

MARION C. WOODS AND MIRIAM E. SIMPSON

Institute of Experimental Biology and Department of Anatomy, University of California, Berkeley and San Francisco, California

ABSTRACT

The action of each of the purified pituitary hormones has been assessed in maintenance and repair of the reproductive system of the hypophysectomized 40-day male rat.

When injections of *interstitial cell stimulating hormone* (ICSH) were begun immediately after hypophysectomy, the testicular weight and physiological status of tubules and Leydig cells were *maintained* and advanced. Testicular epithelium was extremely sensitive to low doses of ICSH and sperm were elaborated. Even at high doses of ICSH, testicular and accessory organ weights were not, however, completely normal for the chronological age. *Follicle stimulating hormone* (FSH), injected at doses which did not show contamination with ICSH, had only slight effects on testicular weight and development; only the highest levels of FSH injected caused barely perceptible stimulation of the accessory organs. However, low doses of FSH injected with ICSH increased testicular size and development. Doses of *lactogenic* and *growth* hormones, having no effect on the reproductive system alone, increased the response to ICSH and also further augmented the response to ICSH with FSH. A dose-response relationship was not demonstrated with increasing doses of either growth or lactogenic hormone. Other anterior pituitary hormones, *thyrotrophic* and *adrenocorticotrophic*, had no influence on the reproductive system given alone or with the gonadotrophins. It should be emphasized that no hormone maintained the testicular tubules of the hypophysectomized male rat unless ICSH was present.

In *repair* of the testis after post-hypophysectomy regression, ICSH did not act as a complete gonadotrophin. Though it repaired the Leydig cells it caused no more than slight repair of the testicular tubules even at high doses. FSH alone was without effect in repair, but was extremely important in cooperative action with ICSH. The combination of ICSH with FSH induced tubular differentiation, sperm appearing in some instances, and augmentation of growth of accessory organs was definite. Addition of either growth or lactogenic hormone to the combination of ICSH with FSH increased the degree of stimulation; simultaneous injection of all four hormones was even more effective, and was the only treatment using purified hormones in which sperm were formed in all animals. (Extracts of rat pituitaries caused similar development after post-hypophysectomy regression.) In repair, as in maintenance of the testis, no combination of pituitary hormones was effective unless ICSH was present.

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FSH acts synergically with testosterone propionate, as it does with ICSH. Precocious sexual maturity in normal weanling rats did not result from injection of purified sheep ICSH and FSH, given with or without supplementation by other pituitary hormones; rat anterior pituitary extracts were similarly ineffective. After hypophysectomy preparations from both heterologous and homologous pituitaries accelerated development of the reproductive tract. The pituitaries of immature male rats contained adequate hormone to induce sexual maturity in hypophysectomized males.

WHEN it had been shown that in the female separate pituitary gonadotrophins were responsible for the two phases of ovarian activity, the growth of follicles and of interstitial cells, it seemed possible that these hormones performed similar functions in the male: namely, that the follicle stimulating factor (FSH) favored the maturation of germinal elements, and that the interstitial cell stimulating factor (ICSH) stimulated the Leydig cells. The latter assumption has received abundant confirmation. The concept that FSH is the chief pituitary factor controlling the seminiferous epithelium still has its advocates, in spite of evidence showing that testicular tubules can be maintained after removal of the pituitary by injection of Leydig cell stimulants or by androgens themselves (1). The testicular tubules have, in fact, been reported to be so sensitive to pituitary ICSH that spermatogenesis could be maintained and advanced in hypophysectomized rats by doses of ICSH which did not maintain the weight of the male accessory organs (2). The interpretation could be made from these combined gametokinetic and androgenic properties that ICSH is the only pituitary gonadotrophin needed in the male; however, certain limitations to the effectiveness of ICSH have been noted. Though sperm develop in the hypophysectomized rat under the influence of ICSH, high doses have not proven effective in increasing weight of the testes at a normal rate; furthermore the accessory organs seldom have attained normal size for the chronological age. The capacity of testosterone to support spermatogenesis was also found to be limited; it was increasingly difficult, for example, to maintain spermatogenesis as the experimental period was prolonged. When the testis was allowed to regress following hypophysectomy, difficulty was experienced in promoting testicular repair by either testosterone or ICSH (3–8). The proposal which is probably most widely acceptable at present is that FSH as well as ICSH is needed for complete development of the male reproductive tract, or that the two hormones act synergically (9–12).

This problem of the hormonal control of the male reproductive system has been approached again with the hope that the greater degree of chemical purity of FSH and ICSH now attained might make it possible to clarify further the respective functions of these two pituitary gonadotrophins. In addition, the other anterior pituitary hormones have been examined for

any action they may have as adjuncts or antagonists to the action of FSH and ICSH on the male reproductive system.

MATERIALS AND METHODS

Animals Used

Hypophysectomized and normal male rats of the Long-Evans strain were employed. In attempts to maintain the reproductive system after hypophysectomy, males approximately 40 days of age (39 to 41 days, ranging in weight from 120 to 160 gms.) were injected for 15 days, beginning on the day of operation. Male rats of this age were well suited to the studies since spermatids with condensed, chromatic nuclei had just appeared in the testis and sperm could reliably be anticipated to appear in the intact rats at 55 days of age. In experimental attempts to repair the male reproductive system after a post-operative period of regression, the rats were again approximately 40 days of age at operation, and 14 days were allowed to intervene between hypophysectomy and onset of therapy. Longer injection periods were necessary in repair than in maintenance studies; injections were therefore continued as long as 28 days.

Hypophysectomy was performed through the parapharyngeal approach. The completeness of hypophysectomy was judged by a number of criteria: body tone, hair quality, size of testis and scrotum; body weight increment before and during the experimental period; terminally, examination of the pituitary site under a binocular dissecting microscope; attention to the vascularity and size of the pituitary target organs, and, in doubtful cases, microscopic examination of thyroids and adrenals.

Method of Injection

Hormonal preparations were injected once daily. ICSH and FSH were dissolved in 0.9% NaCl; ICSH was given either intraperitoneally (IP) or subcutaneously (SC); FSH was injected SC. Growth hormone (GH) and thyrotrophic hormone (TSH) were injected IP in 0.9% NaCl adjusted to pH 8–9. Lactogenic hormone (Lact. H) was also dissolved in saline and was given IP. Adrenocorticotrophin (ACTH) was dissolved in 5% beeswax in sesame oil and injected SC. Testosterone propionate was injected SC in sesame oil.

The standardization and determination of contamination of the hormonal preparations used are listed in the appendix.

RESULTS

Maintenance of the reproductive tract by injection immediately after hypophysectomy

ICSH.—The degree of maintenance of the reproductive system of the hypophysectomized male rat achieved on injection of ICSH is shown in Figures 1–6. The action of ICSH was detectable by increase in testis weight after administration of $\frac{1}{4}$ R.U. daily for 15 days. In terms of unitage this confirms a previous report from this laboratory of the great sensitivity of the tubules to ICSH (2). It should be noted, however, that in the experiment reported here administration of this unitage required only 0.25 μg daily. Higher daily doses, 2 to 5 R.U., were required to maintain the testis within the weight range of the normal testis at onset (40 days of age) and doses as large as 25 R.U. did not increase testicular weights above those characteristic at time of hypophysectomy. The accessory organs of reproduction (ventral prostate and seminal vesicles) regressed almost to hypophys-

FIGS. 1–6. Maintenance of the reproductive system of the hypophysectomized male rat by ICSH. Organ weights (mg.) and physiological responses plotted against log of daily dose in R.U. Rats were hypophysectomized and injections begun at 40 days of age; injection SC or IP 15 days. Bars represent 95% confidence interval ($t_{.05}$–$t_{.95}$) and arrows the range of observations. Figures by each mean represent the number of rats as numerator, the standard error as denominator. Controls: $\overline{H}$ terminal, 15 da. p.o.; normal 40 da. onset, and terminal 55 da.; control values on right.

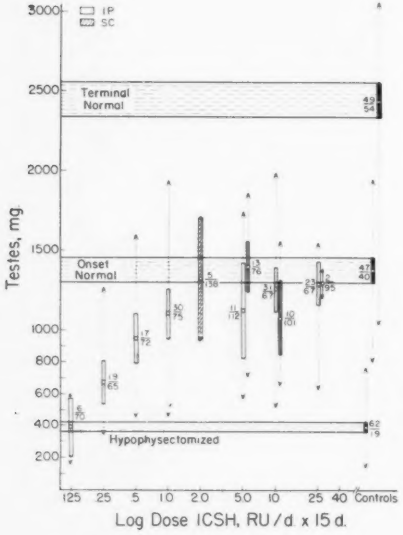


FIG. 1. Weight of testes.

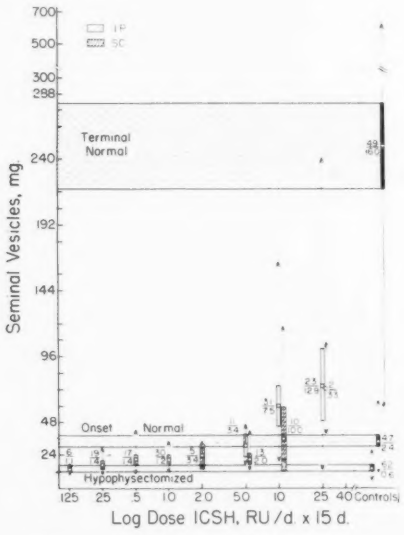


FIG. 2. Weight of seminal vesicles.

ectomized control status in rats receiving low doses sufficient to cause significant stimulation of the testis. Only the highest levels administered (10–25 R.U.) maintained the accessory organ weights within the normal 40 day range or caused moderate further weight increase.

Microscopic examination of the testes showed partial maintenance of the interstitial tissue after administration of $\frac{1}{8}$ to $\frac{1}{4}$ R.U. daily; at higher doses the normal morphology of Leydig cells was maintained or the development advanced to the normal status at 55 days of age. Spermatids with small, chromatic nuclei persisted in testes of some rats receiving the lowest doses ($\frac{1}{8}$ and $\frac{1}{4}$ R.U.), and increasing numbers of spermatids were formed at higher levels. Sperm appeared first in groups receiving $\frac{1}{4}$ R.U. and were present in 70–90% of rats when 5 to 10 R.U. were injected. Sperm therefore formed at the same chronological age as in the normal rat, at 55 days.

Although ICSH accelerated spermatogenesis and advanced spermiogenesis, there was considerable variability among animals both in testicular

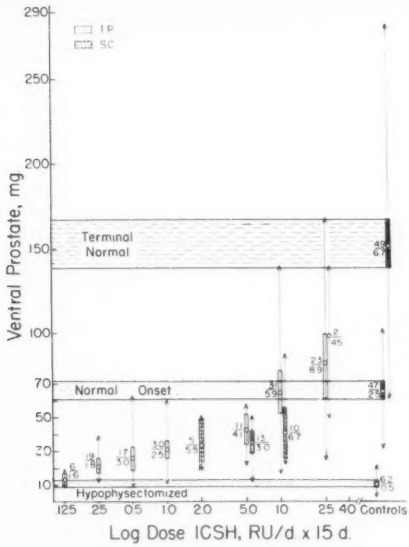


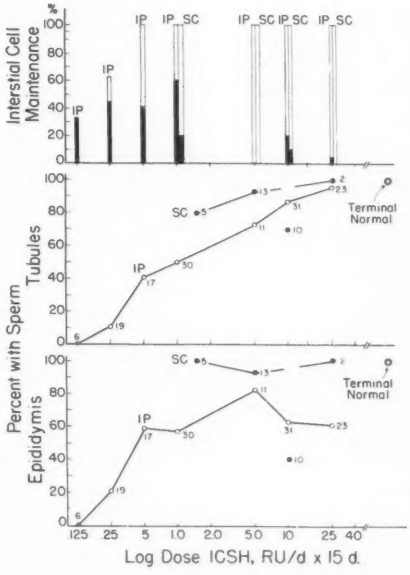
FIG. 3. Weight of ventral prostate.

weights and in the size and development of the seminiferous tubules. The sperm often were not fully mature, and in some animals only small numbers were present. Under the experimental conditions used, ICSH alone

FIG. 4. Percent of rats in which sperm present in epididymis.

FIG. 5. Percent of rats in which sperm present in testis.

FIG. 6. Interstitial cell maintenance; solid bars represent percent of rats showing partial maintenance, open bar maintenance or stimulation.



was therefore not able to advance the reproductive tract at a completely normal rate. The injection period of 15 days was adequate for normal development as will be shown later under other experimental regimens. The route of ICSH injection did not significantly influence either testicular size or tubular development, morphology of Leydig cells, or accessory organ weights (Figs. 2, 3, 6). (In the routine bioassay in the female, ICSH is more potent given IP than SC.) In interpretation of the experimental data it is to be noted that sperm were almost invariably present in testes which had attained the weight of 1000 mg. or more.

FSH.—Purified FSH preparations injected from the time of hypophysectomy did not maintain the reproductive tract when the doses were restricted to those which failed to stimulate interstitial tissue (Figs. 7–10). The testis regressed during the 15 day injection period almost to hypophysectomized control level. Spermatids with condensed chromatin disappeared in animals receiving daily doses of 0.5 to 5 R.U. Some proliferation of spermatogonia continued, and the numbers of spermatocytes in meiosis increased. Division also occurred in some spermatocytes to form young spermatids with vesicular nuclei (recognizable as spermatids by periodic acid Schiff staining of the perforatorium) but further advance in spermiogenesis did not occur (Table 1). Higher levels of FSH (10 to 40 R.U.) maintained testicular weights at onset level or caused slight further growth. Spermatids and sperm were present in an increasing percent of rats receiving doses of 10 R.U. and above. Interstitial cells were atrophic in all groups except the highest doses of FSH (40 R.U.) at which they were only partially maintained. Interstitial tissue did not become functional under the influence of FSH; the weights of seminal vesicles and ventral prostates scarcely exceeded those of hypophysectomized controls after daily doses of 20 to 40 R.U. for 15 days (Figs. 8, 9). It is significant that the lowest level of FSH which caused testicular stimulation (10 R.U.) was known from bioassay to contain about $\frac{1}{4}$ R.U. of ICSH, an amount of ICSH known to be effective in stimulation of testicular tubules. The contamination with ICSH must therefore be kept in mind in evaluating testicular maintenance or continued differentiation such as was observed at high levels of FSH.

ICSH with FSH.—Figures 11–15 show the capacity of ICSH to maintain the male reproductive tract when injected simultaneously with FSH. Increasing doses of FSH were injected, ranging from 0.5 to 5 R.U. At these levels, FSH given alone produced minimal effects on the testis, but when combined with ICSH they markedly increased the capacity of ICSH to stimulate the reproductive tract. Moderate doses of ICSH (1 to 5 R.U.) combined with such levels of FSH advanced testicular weights beyond those of normal onset controls. Higher doses of ICSH (5 to 10 R.U.) combined with FSH overlapped the terminal normal 55 day weight range, whereas neither component of the combination given alone, at any dose, advanced testicular development beyond the 40 day weight range. The

TABLE 1. TESTICULAR AND EPIDIDYMAL DEVELOPMENT IN HYPOPHYSECTOMIZED MALE RATS MAINTAINED BY ICSH, FSH, OR COMBINATIONS THEREOF

Age at H̄ and onset 40 days, injected 15 days

Daily doses (R.U.)		No. of rats	Epididymal tubules (percent of rats)			Testicular tubules (percent of rats)				Leydig cell Maintenance (percent of rats)	
ICSH IP or SC	FSH SC		Enlarged*	Sperm	Debris†	Enlarged	Uniformly enlarged	Sperm	Spermatids	Normal or hypertrophy	Partial (some pyknosis)
0.125		6	0	0	0	17 >H̄	−	0	17	0	33
0.25		19	42 >H	21 few	32	47 >H	−	11	31	16	47
0.5		17	94 >H	59 few	65	94 >H	−	41 few, young‡	82	59	41
1.0		35	83 >H-m	60 few	74	89 =40 da. N	−	57 few, young‡	85	63	37
5.0		27	100 m	89 few	89	100 =40 da. N	−	86 all ages	97	100	
10.0		41	97 m-l	100		100	+	100 all ages	100	100 h	
25.0		28	100 l	100		100	+	100 all ages	100	100 h‖	
	0.5	15	7 >H	0	7	20 >H	−	0	0		0
	1.0	16	31 >H	0	12	44 >H	−	0	0		6
	2.5	18	40 >H	0	50	44 >H	−	0	11 few§		0
	5.0	17	47 >H	0	59	77 >H	−	0	12 few§		35
	10.0	18	94 >H	12 few	89	78 >H	−	30 few, young	68 few§	6	33
	20.0	16	94 >H	19 few	100	100 >H <40 da. N	−	44 few, young	88 few§	0	12
	40.0	8	100 m	75	75	100 >H <40 da. N	+	100 all ages	100	38	38
0.125	0.5	5	67 >H	17	50	67 >H	−	17 few, young	17	17	50
	1.0	5	40 >H̄	40 few	60	40 >H	−	40 few, young	40	20	80
0.25	0.5	23	87 >H̄	26 few	65	74 >H	−	30 few, young	56		66
	1.0	15	93 >H	80 few	100	100 >H	−	80 few, young	93	41	53
0.5	0.5	11	91 >H	73 few	82	91 >40 <55 da.	−	64 few, young	91	36	64
	1.0	17	100 m	82 few	94	88 >40 <55 da.	−	82 few, mature	94	76	24
1.0	0.5	23	100 >H	83 few	87	100 >40 <55 da.	+	87 few, mature	100	43	57
	1.0	24	100 m	92 many	96	100 >40 <55 da.	+	100 all ages	100	77	23
	5.0	11	100 m	83 many	82	91 >40 <55 da.	−	91 all ages	91	100	
5.0	0.5	5	100 m-l	100 many	80	100 ≥40 da. N	+	100 all ages	100	100	
	1.0	32	100 m-l	100 many	81	100 ≥40 da. N	+	100 all ages	100	88	12
	5.0	4	100 l	100 many	100	100 >40 da. N	+	100 all ages	100	75	25
10.0	0.5	10	100 l	70 many	50	100 >40 da. N	−	70 all ages	100	80	20
	1.0	52	100 l	94 many	69	100 >40 da. N	+	100 all ages	100	92	8
	5.0	20	100 l	90 many	55	100 <55 da. N	+	95 all ages	100	90	10
25.0	0.5	5	100 l	60 many	80	100 <55 da. N	+	100 all ages	100	100 h	
	1.0	28	100 l	89 many	64	96 ≈55 da. N	+	96 all ages	100	86 h	14
	5.0	10	100 l	100 many	100	100 ≥55 da. N	+	100 all ages	100	90 h	10

* Detectable enlargement above control (>H); intermediate development and tubules as large as in terminal normals (m, m-l, l).
† Desquamating immature cells from seminiferous epithelium.
‡ Sperm tails delicate and not strongly eosinophilic.
§ Distinguishable though with vesicular nucleus by perforatorium (PAS); increasing numbers of spermatocytes in meiosis and decreasing numbers desquamating as dose increased.
‖ Hyperplasia as well as hypertrophy resulted in groups receiving 25 R.U. ICSH.

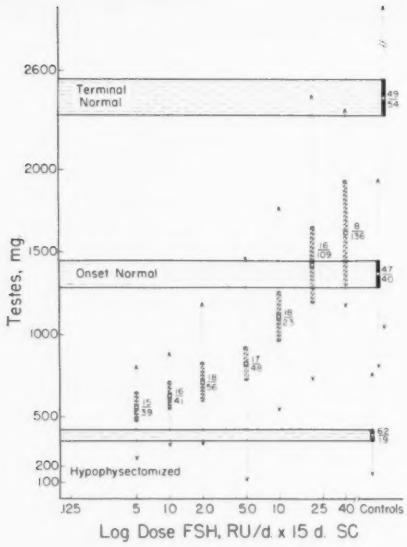


FIG. 7. Weight of testes.

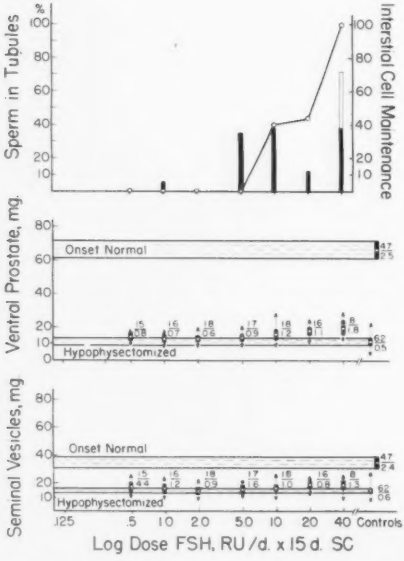


FIG. 8. Weight of seminal vesicles.

FIG. 9. Weight of ventral prostate.

FIG. 10. Line represents percent of rats in which sperm present in testis; the bars represent percent of rats in which Leydig cells were maintained (solid bar represents partial maintenance, open bar maintenance or stimulation).

FIGS. 7–10. Maintenance of the reproductive system of the hypophysectomized male rat by FSH. Organ weight maintenance (mg.) and physiological responses plotted against log of daily dose in R.U. Rats were hypophysectomized and injections begun at 40 days of age; injection SC, 15 days. Bars represent 95% confidence interval and arrows the complete range of observations. Figures by each mean represent the number of rats as numerator, the standard error as denominator; control values on right.

FIGS. 11–15. Maintenance of the reproductive system of the hypophysectomized male rat by combinations of ICSH with increasing doses of FSH (0.5, 1.0, 5.0 R.U.). Organ weights (mg.) and physiological response plotted against log of daily dose of ICSH. Rats were hypophysectomized and injections were begun at 40 days of age; injections continued 15 days. ICSH was either injected IP and FSH given SC, or the combination was injected SC. Bars represent 95% confidence interval and arrows the range of observations. Figures by each mean represent the number of animals as numerator, the standard error as denominator; control values on right.

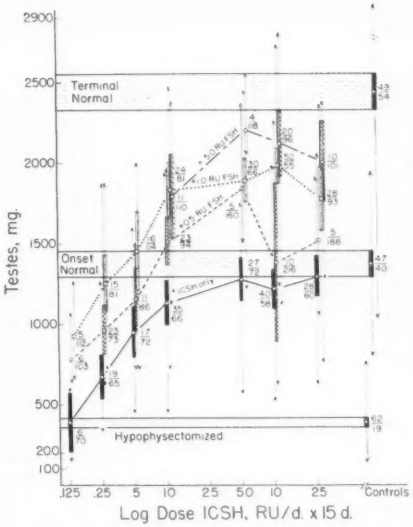


FIG. 11. Weight of testes.

enlargement of seminiferous tubules paralleled the increase in testicular weight. Tubular enlargement and degree of development were more uniform than on injection of ICSH alone. The combination of ICSH with FSH accelerated differentiation, so that spermatids were maintained and spermiogenesis was advanced to sperm formation at lower levels of ICSH than were effective alone (Table 1). Addition of 1 R.U. FSH to doses of ICSH as low as $\frac{1}{8}$ to $\frac{1}{4}$ R.U. (Table 1) resulted in the appearance of sperm in the tubules of 40% and 80% of rats. Sperm were present in greater numbers and were more mature than after injection of ICSH alone, as indicated by increased coarseness and deeper eosinophilia of the sperm tails. The levels of FSH effective in such combinations were so low, and so inactive by themselves, that the effects cannot be considered additive; the increased response is therefore called synergism or augmentation.

Only at the lowest levels of ICSH ($\frac{1}{8}$ to $\frac{1}{4}$ R.U.) injected with FSH was there a suggestion of augmentation of interstitial cell stimulation (Fig. 15). The weights of accessory organs of animals receiving 10 to 25 R.U. ICSH combined with FSH were brought within normal terminal 55-day weight range, a result not attained by ICSH alone (Figs. 12, 13), but the response was so variable that it could not be said with certainty that the accessory organ weights were augmented. No significance could be attributed to route of injection of ICSH, the results being similar when ICSH was given IP and when it was combined *in vitro* with FSH and injected SC.

GH injected with ICSH, or ICSH and FSH.—In order to test the effect of simultaneous administration of GH, a preparation potent at 40 μg was

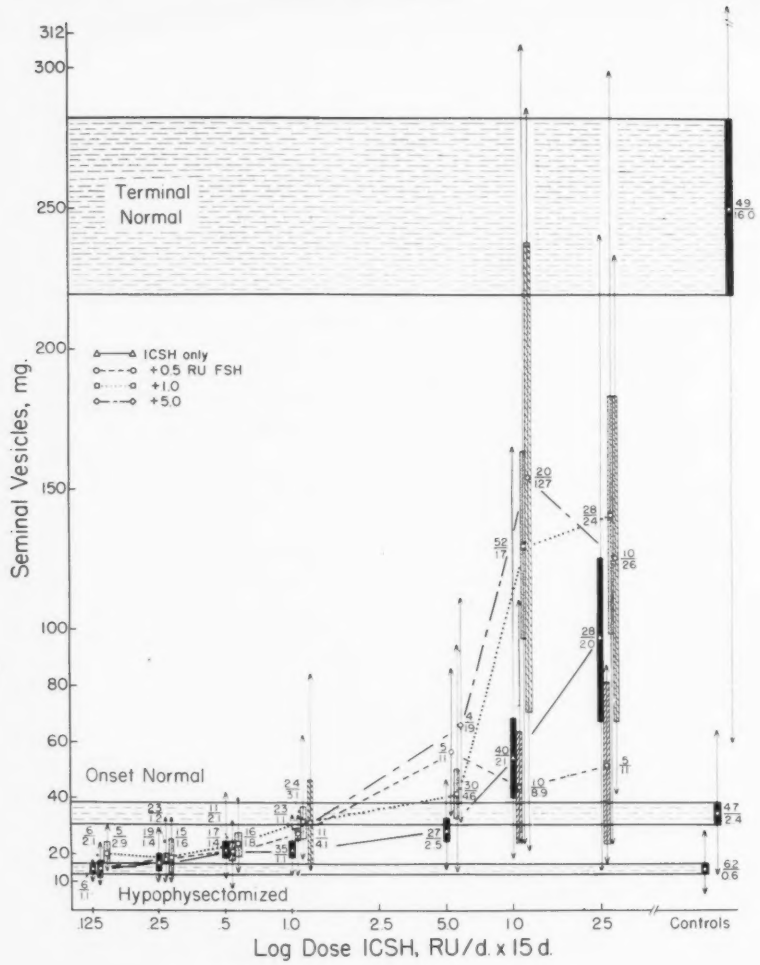


FIG. 12. Weight of seminal vesicles.

injected daily in doses of 2.5 R.U. combined with ICSH. The GH alone did not prevent testicular regression to hypophysectomized status. ICSH combined with GH resulted in testicular weights equal to or greater than the upper range of normal onset controls, a result which was infrequently achieved by ICSH alone (Figs. 16–21). The two hormones were injected by different routes, SC and IP, so that the increased response could not be attributed to an effect of GH on rate of absorption of ICSH. The beneficial results could not be attributed to ICSH contamination in GH inasmuch as the maximal daily injection, 2.5 R.U., contained no more than 0.04 R.U. ICSH, and doses as low as 0.6 and 1.2 R.U. efficiently augmented ICSH (Table 2). The effectiveness of combinations of ICSH with FSH was

TABLE 2. MAINTENANCE OF THE REPRODUCTIVE SYSTEM IN HYPOPHYSECTOMIZED MALE RATS BY ICSH ENHANCED BY INCREASING DOSES OF GH

Age at H̄ and onset 40 days, injected 15 days

Daily doses (R.U. 15 da.)		No. of rats	Organ weights (mg. ± standard error)			Testicular response (% of rats)		Adrenals (mg. ± S.E.)	Tibial epiphyseal cartilage (μ. ± S.E.)	B.W. change (gm./15 da.)
ICSH SC	GH IP		Testes	Ventral prostate	Seminal vesicles	Sperm present	Leydig cells epithelioid			
5	0	27*	1280 ± 72	41 ± 8.0	28 ± 2.5	86	109	11 ± 0.4		−18
		6	1450 ± 174	30 ± 5.8	22 ± 3.2	100	100		143 ± 2.7	−21
	0.6	4	1629 ± 106	67 ± 3.9	67 ± 5.9	100	100		247 ± 5.7	0
	1.2	8	2044 ± 193	85 ± 9.2	72 ± 12	100	100	16 ± 1.1†	204 ± 8.7	+ 6
	2.5	13	1610 ± 106	79 ± 5.3	80 ± 11	94	100	17 ± 2.6†	228 ± 10.0	+ 9
Controls H̄ 40 da. 15 da. p.o.		62	386 ± 19	11 ± 0.5	14 ± 0.6	0	0	9 ± 0.9	150 ± 4.0	−16
N onset 40 da.		47	1372 ± 40	66 ± 2.5	34 ± 2.4	0	100 few			
N terminal 55 da.		49	2448 ± 54	153 ± 6.7	250 ± 16	100	100			+74

* Composite figure for all rats receiving this level.

† Adrenals in these groups showed only microscopic evidence of minimal stimulation (+); the thyroids showed no weight increase, and no microscopic evidence of stimulation at any level except a marginal reaction at 2.5 R.U.

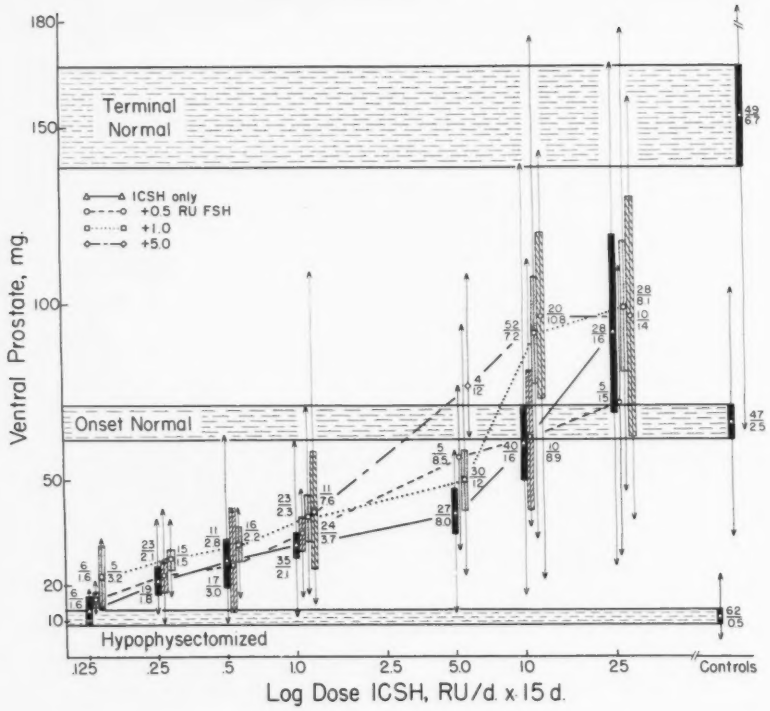


FIG. 13. Weight of ventral prostate.

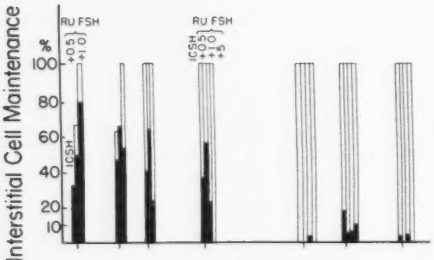


FIG. 14. Percent of rats in which sperm were present.

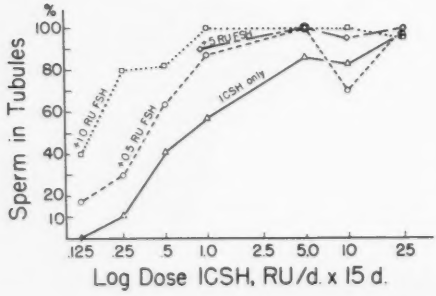


FIG. 15. Leydig cell maintenance is represented by bars; solid bars represent partial maintenance, open part maintenance or stimulation.

further increased by addition of GH, raising testis weights to the range of terminal 55 day normals (Figs. 16–21).

The increased accessory organ weights were even more striking than the increased testicular weights after combination of GH with ICSH, or ICSH and FSH. The ventral prostate was only maintained at onset weight by injection of 10 and 25 R.U. ICSH, and after addition of FSH was still below normal terminal average weight, but on addition of GH either to ICSH, or ICSH with FSH, prostatic weights were raised to the normal 55-day range. The seminal vesicles showed essentially the same type of response but due to accumulation of extensive fluid the weights were more variable (Fig. 17).

Although the 2.5 R.U. level of GH injected gave no evidence of ICSH contamination, traces of ACTH, TSH and Lact. H were known to be present (see appendix and Table 2). Periodate treatment of the preparation reduced the contamination still further, but the biological results in males were the same and have not been listed separately. However, as the augmentation of the action of ICSH by GH was so definite and since biologically active contaminants were present, it seemed important to determine the effect of each of the three contaminating pituitary hormones under the present experimental conditions.

ICSH, or ICSH with FSH, injected with ACTH or with TSH.—Purified preparations of ACTH and TSH were injected in hypophysectomized male rats alone and in combination with ICSH, or ICSH with FSH. The doses of ACTH and TSH chosen were low, as the objective was to determine whether doses approximating those present as contaminants in the GH would exert an influence. ACTH administered in doses of 0.5 to 2.5 U.S.P. units per day did not maintain the weights of testes or accessory organs when given alone (Table 3). The action of these doses of ACTH on the adrenal cortex was similar to, or greater than, that produced by the GH, yet did not augment or inhibit the effect of ICSH alone, or ICSH with FSH. Similarly, 0.1 U.S.P.U. of TSH, which was capable of eliciting a morphological response in the thyroid equivalent to or greater than that exerted by the GH preparation, did not influence the reaction from administration of ICSH alone, ICSH with FSH, or ICSH combined with FSH and GH (Table 3).

Although not included in Table 3, sodium L-thyroxine was also injected at doses of 1 μg daily. Thyroxine given alone or combined with 2.5 R.U. GH exerted no effect on the gonadotrophic potency of ICSH, or of ICSH with FSH. The dose of thyroxine selected was known to elevate basal metabolic rate significantly, and, in combination with GH, to restore calorigenesis in the hypophysectomized rat to normal or above (13). It appears safe to conclude that the augmentation of the gonadotrophic potency of ICSH by GH is not due to contamination with either ACTH or TSH.

ICSH, or ICSH and FSH, injected with Lact. H.—Lact. H was found to

TABLE 3. FAILURE OF PURIFIED PREPARATIONS OF ACTH AND TSH TO ENHANCE MAINTENANCE OF THE REPRODUCTIVE SYSTEM IN HYPOPHYSECTOMIZED MALE RATS BY ICSH, OR ICSH WITH FSH

Age at H and onset 40 days, injected 15 days

Daily doses (15 da.)					No. of rats	Organ weights (mg. ± standard error)			Testicular response (percent of rats)		Adrenals		Thyroid response	Tibial epiphyseal cartilage (μ ± S.E.)	B.W. change (gm., 15 da.)
ICSH (R.U.) SC	FSH (R.U.) SC	ACTH U.S.P.U. SC	TSH U.S.P.U. IP	GH (R.U.) IP		Testes	Ventral prostate	Seminal vesicles	Sperm present	Leydig cells epithelioid	mg. ± S.E.	Response			
		0.5			5	378 ± 60	11 ± 1.6	12 ± 1.5	0	0	13 ± 0.6	+			−14
		1.5			5	488 ± 61	13 ± 1.7	20 ± 1.5	0	0	17 ± 0.8	+ to + + +			− 9
		2.5			5	501 ± 56	12 ± 1.5	16 ± 2.8	0	0	30 ± 1.9	+ + to + + +			−30
5					27	1280 ± 72	41 ± 8.0	28 ± 2.5	86	100	11 ± 0.4	—			−18
5		1.5			5	1416 ± 119	31 ± 5.0	18 ± 2.8	100	100	19 ± 1.7	+ to + + +			−14
5	1.0				30	1895 ± 66	50 ± 12	41 ± 4.6	100	97	11 ± 0.5	− (±)			−15
5	1.0	1.5			5	1740 ± 183	35 ± 3.2	23 ± 2.1	100	100	19 ± 1.2	+ + to + + +			−26
5	1.0	2.5			9	2000 ± 92	35 ± 4.4	23 ± 2.4	100	78	33 ± 1.5	+ + to + + +			−25
5			0.1		4	1446 ± 169	26 ± 3.3	22 ± 2.5	100	100	10 ± 0.9	−	+	153 ± 0.9	−20
5	1.0		0.1		10	2020 ± 83	42 ± 3.6	30 ± 3.6	100	80			+ to + +		−16
5	1.0		0.1	2.5	10	2265 ± 37	82 ± 6.1	70 ± 7.9	100	100		±	+	251 ± 18	+22
5	1.0			2.5	20	2299 ± 159	107 ± 10	119 ± 14	100	100		+	±	222 ± 22	+11
Controls															
H 40 da. 15 da. p.o.					62	386 ± 19	11 ± 0.5	14 ± 0.6	0	0	9 ± 0.9			150 ± 4.0	−16
N onset 40 da.					47	1372 ± 40	66 ± 2.5	34 ± 2.4	0	100					
N terminal 55 da.					49	2448 ± 54	153 ± 6.7	250 ± 16	100	100					±74

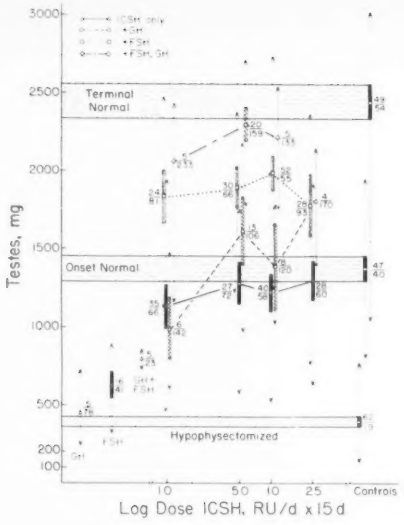


FIG. 16. Weight of testes.

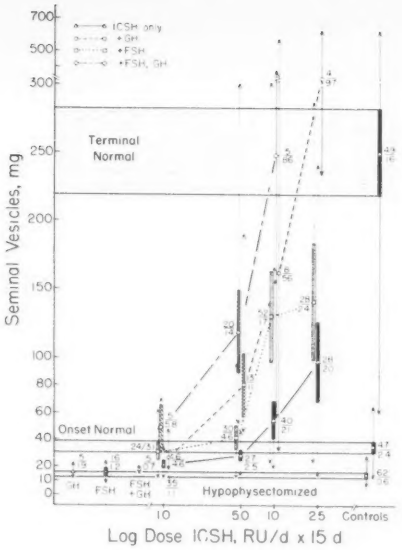


FIG. 17. Weight of seminal vesicles.

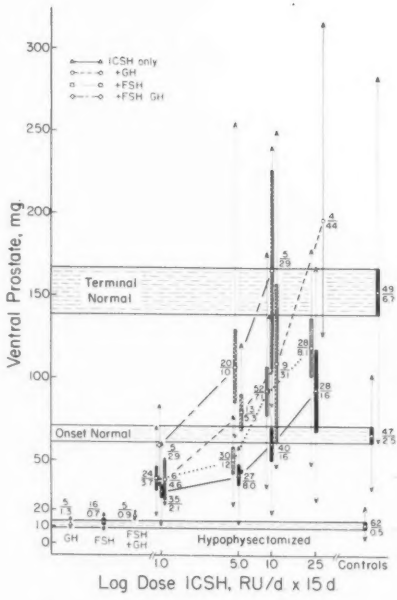


FIG. 18. Weight of ventral prostate.

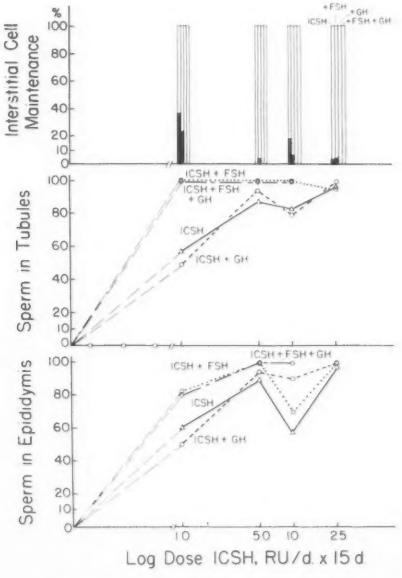


FIG. 19. Percent of rats in which sperm present in epididymis.

FIG. 20. Percent of rats in which sperm present in testicular tubules.

FIG. 21. Percent of rats in which Leydig cells maintained; solid bar partial maintenance; open portion maintenance or stimulation.

FIGS. 16–21. Effect of the addition of GH to ICSH, or to ICSH+FSH, on maintenance of the reproductive system of the hypophysectomized male rat. Organ weights (mg.) and physiological responses plotted against log of daily dose of ICSH. Constant daily doses of FSH (1 R.U.) and GH (2.5 R.U.) were injected; their effects given alone are included. Hypophysectomy 40 da. of age; injections begun at once and continued 15 da. GH was injected IP, all other hormones SC. Bars represent 95% confidence interval and arrows the range of observations. Figures by each mean represent the number of rats as numerator, the standard error as denominator; control values on right.

be a significant factor, however, in augmentation of the response to ICSH. The role of Lact. H was therefore more carefully studied in a dose range from 0.3 to 10 I.U. Preparations of Lact. H, though possessing no intrinsic power to increase testicular weights in this dose range, did increase significantly the response both to ICSH and ICSH with FSH (Figs. 22–24 and Table 4). Addition of Lact. H to ICSH did not result in testicular weights as great as observed on addition of FSH to ICSH; nevertheless, Lact. H added to the combination of FSH with ICSH was able to cause an even

TABLE 4. MAINTENANCE OF THE REPRODUCTIVE SYSTEM OF THE HYPOPHYSECTOMIZED MALE RAT BY ICSH ALONE, OR WITH FSH, ENHANCED BY LACTOGENIC HORMONE

Age at H and onset 40 days, injected 15 days

Daily doses (15 da.)					Organ weights (mg.± standard error)			Testicular response (percent of rats)		Tibial epiphyseal cartilage (μ± S.E.)	B.W. change (gm.)
Lactogenic hormone IP		ICSH SC or IP (R.U.)	FSH SC (R.U.)	No. of rats	Testes	Ventral prostate	Seminal vesicles	Sperm present	Leydig cells epithelioid		
Preparation	I.U.										
NIH-P-S-3 15 I.U./mg.	2			4	446± 70	14± 2.6	18± 1.9	0	0		−25
	5			5	332± 74	11± 1.2	12± 0.9	0	0		−18
	10			5	368± 20	12± 0.7	13± 1.1	0	0		−21
		5		27*	1280± 72	41± 8.0	28± 2.5	86	100		−18
				6	1450± 174	30± 5.8	22± 3.2	100	100	143± 2.7	−21
	0.5	5		5	1728± 271	86± 4.1	65± 19	100	100	166± 4.2	−12
	1.0	5		5	1726± 147	68± 4.1	58± 12	100	100	156± 7.7	−27
	2	5		8	1583± 142	116± 16	150± 39	100	100	185± 12	− 6
	5	5		5	1528± 169	132± 26	156± 46	80	100		− 6
		5	1.0	30*	1895± 66	50± 12	41± 4.6	100	97		−15
				4	2058± 129	63± 13	54± 16	100	100		− 9
	2	5	1.0	5	2238± 116	131± 24	253± 53	100	100		− 8
	5	5	1.0	2	2206± 50	132± 19	214± 118	100	100		−12
			1.0	16	638± 41	12± 0.7	16± 1.2	0	0		−13
	5		1.0	4	704± 46	13± 0.6	16± 1.8	0	0		−19
L2378A 15–20 I.U./mg.		5		5	1669± 96	44± 5.5	30± 7.0	100	100	139± 7.4	−21
	0.3	5		5	2037± 148	62± 8.0	40± 6.6	100	100	138± 3.2	−15
	0.6	5		5	1766± 156	67± 6.1	55± 18	100	100	136± 2.0	−15
	1.2	5		4	2037± 119	86± 14	62± 14	100	100	160± 4.2	−17
	3	5		6	1887± 117	79± 9.0	78± 16	100	100	157± 4.8	−15
Controls H 40 da. 15 da. p.o.				62	386± 19	11± 0.5	14± 0.6	0	0	150± 4.0	−16
N onset 40 da.				47	1372± 40	66± 2.5	34± 2.4	0	100 few		
N terminal 55 da.				49	2248± 54	153± 6.7	250± 16	100	100		+74

* Composite figure for all rats receiving this level.

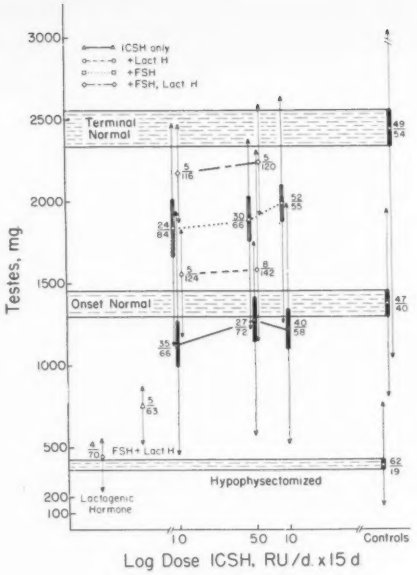


FIG. 22. Weight of testes.

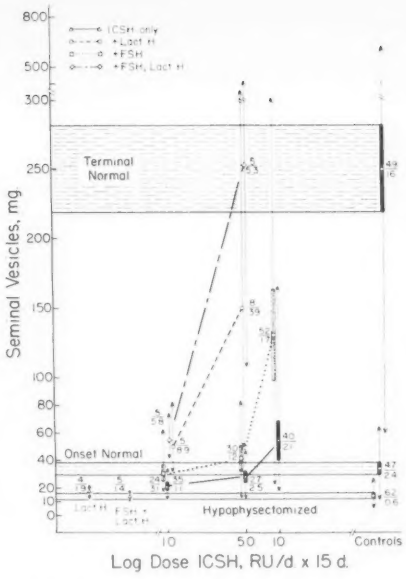


FIG. 23. Weight of seminal vesicles.

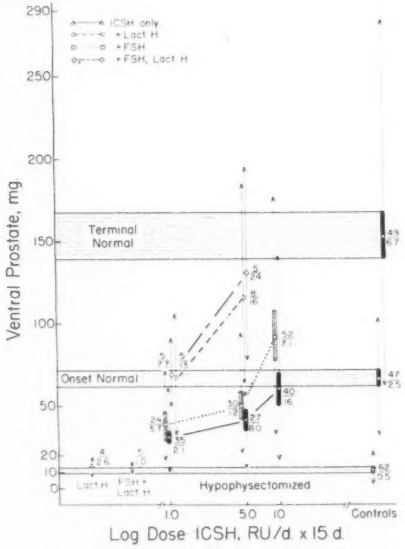


FIG. 24. Weight of ventral prostate.

FIGS. 22–24. Effect of the addition of Lact. H to ICSH, or to ICSH+FSH, on maintenance of the reproductive system of the hypophysectomized male rat. Organ weights (mg.) plotted against log of daily dose of ICSH. Constant unitages of FSH (1 R.U.) and Lact. H (2 I.U., NIH-P-S-3) were employed as shown. Rats were hypophysectomized and injections begun at 40 days of age and continued 15 days. Lact. H was given IP; ICSH alone, IP; combination ICSH with FSH, SC. Bars represent 95% confidence interval and arrows the range of observations. Figures by each mean represent the number of rats as numerator, the standard error as denominator; control values on right.

greater testicular weight response (Fig. 22). The maximum augmentation by Lact. H could be observed at the lowest dose injected (0.5 I.U.); as can be noted in Table 4 no further increase occurred on injection of 10-fold this dose. The accessory organ weights also were not definitely greater at higher dose levels of Lact. H (Table 4). As in the case of GH no dose-response relationship could satisfactorily be demonstrated.

Repair of the male reproductive tract after regression following hypophysectomy

The repair of the reproductive system after post-hypophysectomy regression constitutes a more rigorous test of hormonal needs of the testis than supportive therapy instituted immediately after hypophysectomy. A parallel investigation of the factors needed for repair was therefore conducted, including a study of the action of ICSH, alone or combined with FSH, GH, and Lact. H. Figures 25–27 and Table 5 record the repair induced by various combinations of these hormones when injections were begun 14 days after hypophysectomy at 40 days of age and were continued for 15, 21 or 28 days. Either ICSH or FSH given separately in doses higher than those required in maintenance studies, caused only slight increase in testicular weight and only limited improvement in the testicular epithelium. Although ICSH did not appreciably increase testicular tubule size, vigorous cellular proliferation occurred; spermatocytes in meiosis and a few spermatids with compact, deeply basophilic nuclei appeared within 15 days. However, differentiation did not advance further on continued injection for an additional 2 weeks.

FSH caused even less repair of testicular tubules than ICSH; spermatocytes were increased in number; a few undertook meiosis, and very young spermatids were detectable after 15 and 21 days. This activity was not sustained, and by 28 days no spermatids remained and spermatocytes were again depleted, leaving only a few desquamating spermatocytes such as are observed in the hypophysectomized rat. It was not clear that FSH given alone could make a significant contribution to testicular growth or differentiation. The importance of FSH in repair of the degenerate testis was amply demonstrated, however, when FSH was injected with ICSH. This combination actually caused reinstatement of testicular weights and development to the 40-day normal range after a 21-day injection period. More important still, sperm began to appear after 15 days, and within 21 to 28 days sperm were present in 40–60% of the rats. The addition of GH to the mixture of FSH and ICSH caused no further improvement in spermatogenesis.

ICSH reinstated the deficient interstitial cells within 15 days, although some regression in the response occurred on more prolonged administration. The doses of FSH injected alone caused no morphological change in the deficient interstitial cells. Only moderate stimulation of interstitial tissue function resulted from injection of ICSH alone, reinstatement of the 40

TABLE 5. REPAIR OF THE REPRODUCTIVE SYSTEM OF THE HYPOPHYSECTOMIZED MALE RAT BY ICSH, OR ICSH AND FSH, SUPPLEMENTED BY GROWTH AND LACTOGENIC HORMONES

Age at H 40 days and onset 14 days postoperative

Days injected	Daily doses: ICSH (R.U.) SC	FSH (R.U.) SC	GH (R.U.) IP	Lact. H. L2387A (I.U.) IP	No. of rats	Organ weights (mg. ± standard error): Testes	Ventral prostate	Seminal vesicles	Testicular response (percent of rats): Sperm	Spermatids (compact nuclei)	Leydig cell repair*	Adrenals (mg. ± S.E.)	Thyroid response	Tibial epiphyseal cartilage (μ ± S.E.)	B.W. change injection period‡ (gm.)
15	15				7	556 ± 54	26 ± 3.7	29 ± 3.3	0	25	100	12 ± 1.6†			+ 5
		5			4	535 ± 34	9 ± 0.2	10 ± 0.1	0	0	0				+ 2
	15	5			7	984 ± 137	36 ± 2.2	53 ± 20	15	85	100				+ 5
	25	5			4	1198 ± 110	88 ± 22	187 ± 69	0	100	100				+ 4
21	15				6	402 ± 64	24 ± 8.6	32 ± 7.0	0	33 few	40 (60 partial)				− 1
		5			6	532 ± 61	10 ± 0.5	14 ± 1.8	0	0*	0				− 4
	15	5			6	1392 ± 157	66 ± 19	243 ± 81	67	100	100				+ 4
	15		2.5		5	512 ± 111	48 ± 18	87 ± 45	20	40	40 (20 partial)	9 ± 1.0		191 ± 9.0	+30
	15	5	2.5		5	1066 ± 62	102 ± 23	205 ± 60	40	100	100	10 ± 1.7		218 ± 20	+34
28	15				10	494 ± 71	21 ± 5.6	31 ± 7.8	0	50 few	40 (30 partial)		—	135 ± 2.6	+10
		5			5	286 ± 19	9 ± 0.9	10 ± 0.9	0	0*	0				+ 8
	15	5			10	885 ± 138	35 ± 12	71 ± 34	40	60	50 (10 partial)		−(±)	144 ± 6.5	+11
	15		2.5		5	596 ± 72	95 ± 5.6	196 ± 95	0	80	80		±(+)	245 ± 5.0	+67
	15	5	2.5		6	1052 ± 144	78 ± 22	195 ± 73	50 few, young	83	100		+	255 ± 11	+61
	15			1.5	5	606 ± 122	43 ± 20	34 ± 10	20 few, abnormal	80	80			144 ± 3.4	+12
	15	5		1.5	10	962 ± 115	31 ± 5.4	41 ± 6.0	40	90	100		±	132 ± 5.7	+15
	15	5	2.5	1.5	5	1315 ± 69	88 ± 22	141 ± 72	100	100	100		−(±)	219 ± 17	+55
Controls															
H 14 da. p.o. (onset)					62	386 ± 19	11 ± 0.5	14 ± 0.6	0	0	atrophic	9 ± 0.9	—	150 ± 4	−16
H 29 da. p.o. for 15 da. injection					2	232 ± 35	8 ± 0	14 ± 4	0	0	atrophic	9 ±	—	119 ± 12	−19
H 35 da. p.o. for 21 da. injection					2	264 ± 42	8 ± 0	11 ± 1	0	0	atrophic		—	105 ± 9	−20
H 42 da. p.o. for 28 da. injection					3	242 ± 35	8 ± 0.9	14 ± 2.8	0	0	atrophic		—	120 ± 7	−16
N 40 da.					47	1372 ± 40	66 ± 2.5	34 ± 2.4	0	100					
N 55 da.					49	2448 ± 54	153 ± 6.7	250 ± 16	100	100					+74

* After injection of FSH for 7 days tubules within H control limits; 14 da. few more adherent spermatocytes, even early spermatids, perforatorium stained by PAS. 21 da. some slightly improved beyond 15 da.; 28 da. regression to H control condition.

† Minimal histological response, all others negative.

‡ Only those rats receiving growth hormone recovered pre-injection loss.

FIGS. 25–27. Effect on repair of the reproductive system of the hypophysectomized male rat of addition of GH and/or Lact. H to ICSH, or ICSH+FSH. Organ weights (mg.) plotted against period of injection (15, 21 or 28 days). Hypophysectomy was performed at 40 da. and onset of injection was 14 da. later. The daily dosage was ICSH 15 R.U., SC; FSH 5 R.U., SC; GH 2.5 R.U., IP; Lact. H 2 I.U., IP. Bars represent 95% confidence interval and arrows the range of observations. Figures by each mean represent the number of rats as numerator, the standard error as denominator; control values on right; terminal $\overline{H}$ values enclosed in transverse bars.

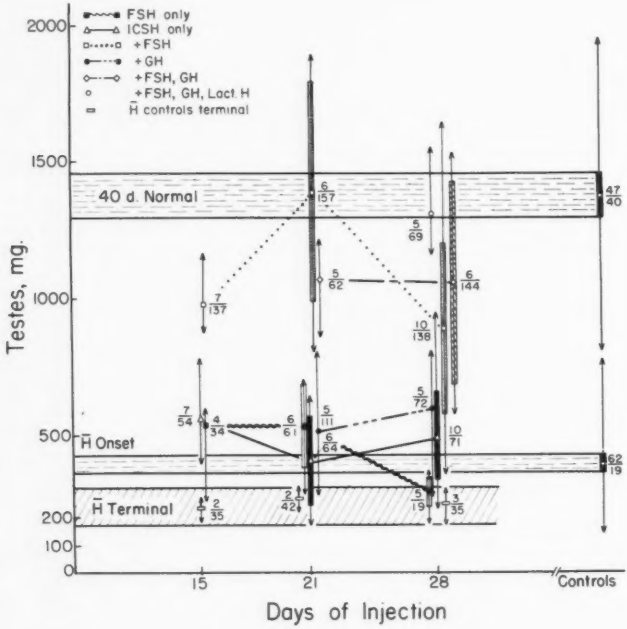


FIG. 25. Weight of testes.

day old normal weights of the ventral prostate being attained. (Only limited functional response to ICSH had also been characteristic in the maintenance experiments.) Administration of FSH with ICSH definitely increased the functional response resulting in accessory weights equal to, or greater than, those in normal 40-day-old rats. The augmentation of accessory organ response to ICSH on combination with FSH was far more definite in repair than in maintenance experiments. The accessory organs reached their maximal size after injection of ICSH with FSH for 21 days, and after 28 days there was some evidence of regression.

Injection of GH with ICSH increased the repair of the accessory organs to a degree comparable to that following injection of FSH with ICSH. Addition of GH to combinations of ICSH with FSH resulted in stimulation in excess of that resulting from any two of these substances given in com-

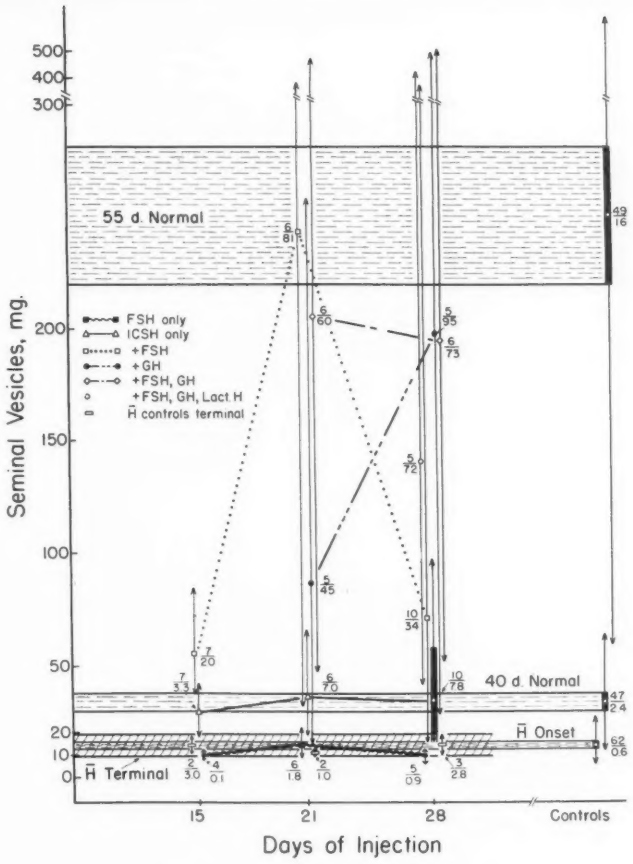


FIG. 26. Weight of seminal vesicles.

bination; the weights of accessory organs of rats subjected to the triple stimulus were intermediate between those of 40- and 55-day-old normal males.

The addition of Lact. H to combinations of ICSH, FSH and GH did not significantly improve the weights of accessory organs or of the testes but did improve spermatogenesis; administration of the quadruple mixture was in fact the only treatment with purified hormones able to repair the testis and advance development to sperm formation in 100% of the rats.

Maintenance and repair of the reproductive tract by rat anterior pituitary

The efficacy of purified sheep hormones in maintenance and repair of the male rat reproductive system after hypophysectomy was compared with that of rat anterior pituitary extracts (Table 6). These extracts adminis-

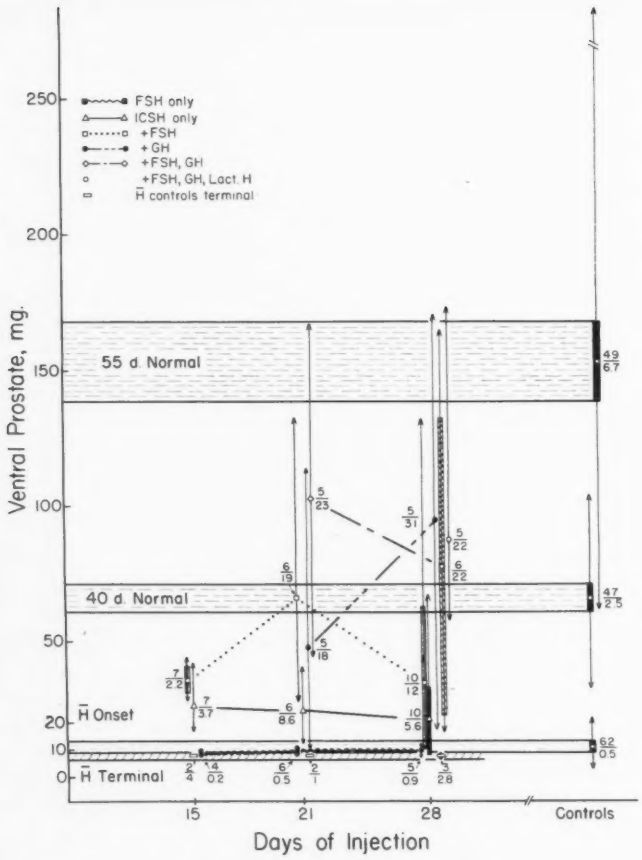


FIG. 27. Weight of ventral prostate.

tered immediately after hypophysectomy in unitages as similar as possible to effective unitages of sheep pituitary gonadotrophins (i.e., having the same follicle stimulating and interstitial cell stimulating potency) caused development in testes and accessory organs at a rate equal to or in excess of that normal for the chronological age (55 days). When a 14-day period of regression had elapsed before treatment with rat pituitary extracts was begun, these unitages repaired the atrophied testicular tubules and advanced spermatogenesis and spermiogenesis to sperm production within 21-days. The weights of the testes did not exceed those of 40-day rats although the weights of accessory organs were comparable to those of 55 day normal rats. These results from injection of extracts of rat pituitaries closely paralleled those from the quadruple mixture of purified sheep pituitary hormones.

TABLE 6. MAINTENANCE AND REPAIR OF THE REPRODUCTIVE SYSTEM OF HYPOPHYSECTOMIZED MALE RATS BY RAT PITUITARY EXTRACTS

Age of recipients at			Rat pituitary daily dose, SC					No. of rats	Organ weights (mg.± standard error)			Testicular response (percent of rats)			Adrenals		Thyroid response	Tibial epiphyseal cartilage (μ± S.E.)	B.W. change (gm.)
				Daily dose in equivalents of															
(da.)	Onset (da.)	Autopsy (da.)	Type of preparation	ICSH (R.U.)	FSH (R.U.)	GH (R.U.)	Days injected		Testes	Ventral prostate	Seminal vesicles	Sperm	Spermatids (compact nuclei)	Leydig cells stimulated	mg.± S.E.	Response			
	Maintenance 0 da. p.o.	55	40% EtOH extr., adult, ♀, ♂	8	2	ca 1	15	4	2734± 141	216± 9.4	717± 170	100 mature	100	100	15± 1.0	+ to ++	++ to +++	200± 17	−11
40	Repair 14 da. p.o.	69	Same	15	2	2	15	4	1104± 142	171± 15	376± 106	0	75	100	12± 0.4	+ to ++	+ to ++	244± 20	+21
		75	40% EtOH extr., adult, castrates, ♂	15	2	2	21	5	1284± 83	183± 36	368± 119	100 young	100	100		+ to ++			+20
—	Normal, 21 da.	42	Same	20	3	ca 2	15	5	1370± 47	135± 4.1	45± 5.6	0	100	100					+64
23	Maintenance 0 da. p.o.	44	Same	20	3	ca 2	21	6	1650± 80	134± 13	123± 21	100 few, young	100	100	8± 1.3	—	++ to +++	237± 14	+21
40	Maintenance 0 da. p.o.	55	Homogenate 23–33 da. ♂	0.8 gland			15	3	2491± 243	137± 28	186± 57	100 mature	100	100	18± 2.0	+	++ to +++	274± 3.2	+18
Controls																			
23	—	44						1	58	6	7	0	0	0	4	—	—	133	− 3
40		54						62	386± 19	11± 0.5	14± 0.6	0	0	0	9± 0.9	—	—	150± 4.0	−16
40		69						2	232± 35	8± 0	14± 4	0	0	0		—	—	119± 12	−19
40		75						2	261± 42	8± 0	11± 1	0	0	0		—	—	105± 9	−20
—	Normal	23						3	281± 22	25± 0.6	8± 1.3	0	0	0	21± 2.0				
—	Normal	40						47	1372± 40	66± 2.5	34± 2.4	0	100						
—	Normal	55						49	2448± 51	153± 6.7	250± 16	100	100						

Hormonal factors necessary for reproductive system development in weanling male rats

Purified sheep gonadotrophins in normal and hypophysectomized weanling rats.—It is well known that gonadotrophic agents, effective in promoting precocious puberty in the female, fail to promote precocious puberty in the immature male (14, 15). It would constitute a contribution to the understanding of the hormonal control of the testis if an explanation could be found for disappearance of this insensitivity of the testis after hypophysectomy. An attempt has been made to apply to the intact immature rat the information gained regarding the hormonal requirements for maintenance and repair of the testis of the hypophysectomized rat. Normal weanling males (21 to 23 days of age, weighing 60 to 75 gm.) were injected for a 21-day period with purified sheep pituitary gonadotrophins. The age at termination of the experiment was therefore 40 to 44 days, an age at which the appearance of spermatids in untreated controls could be assured. The testes of the weanlings were characterized by small tubules, compactly filled by a many-layered epithelium which included enlarging spermatocytes in various phases of meiosis. The interstitial cells were sparse, very few having become enlarged and epithelioid. ICSH was injected at doses of 25 R.U., a level far greater than necessary to induce a response in the much larger males hypophysectomized at 40 days of age. During the 21-day injection period the testes increased in weight at a somewhat subnormal rate, but the accessory organs attained weights normal for the age. A few spermatids with compact chromatin appeared in the testicular tubules. No significant modification was therefore introduced by the ICSH treatment in the normal course of tubular development (Table 7). In the group injected with FSH alone, all organ weights were slightly subnormal for the age and no spermatids were formed. The combination of ICSH with FSH did not increase the weights of testes or seminal vesicles in excess of the normal range; only the weight of the ventral prostate exceeded normal. These experiments, therefore, confirm previous reports of the unresponsiveness of the normal immature rat testis to exogenous pituitary gonadotrophins.

When rats were hypophysectomized at this early age (23 days) and were injected immediately after operation with lower doses of ICSH and FSH than those given normal rats, testicular growth occurred at almost the normal rate (Table 7). Spermatogenesis was accelerated beyond normal rate and sperm were formed in a large proportion of the rats by 44 days of age. The interstitial tissue became hypertrophic and, in some instances, even moderately hyperplastic. The growth rate of seminal vesicles and ventral prostate equalled or slightly exceeded the rate in normal rats. Neither growth nor lactogenic hormone improved the growth and differentiation of the reproductive system of hypophysectomized weanlings injected with purified sheep ICSH and FSH.

TABLE 7. RESPONSE OF THE REPRODUCTIVE SYSTEM IN NORMAL AND HYPOPHYSECTOMIZED WEANLING MALE RATS TO SHEEP PITUITARY GONADOTROPHINS

Type of recipient	Daily dose (21 da.)				No. of rats	Organ weights (mg. ± standard error)			Testicular response (percent of rats)		
	ICSH (R.U.) SC	FSH (R.U.) SC	GH (R.U.) IP	Lact. H NIH-P-S-3 (I.U.) IP		Testes	Ventral prostate	Seminal vesicles	Sperm	Spermatids (compact nuclei)	Leydig cell stimulation
N Onset 21 da.	25				5	888 ± 34	113 ± 20	31 ± 5.8	0	80 few	epithelioid
Autopsy 42 da.		10			5	1028 ± 91	55 ± 5.9	22 ± 3.3	0	0*	epithelioid
	25	10			5	1430 ± 91	131 ± 3.0	57 ± 7.5	0	100 many	epithelioid
H̄ 23 da.	15	5			5	1273 ± 108	88 ± 20	61 ± 22	80	100	hyperplasia
Onset 23 da.	15	5	2		1	1314	90	47	+	+	hyperplasia
Autopsy 44 da.	15	5		2	6	1074 ± 109	126 ± 15	94 ± 21	80	100	hyperplasia
Controls											
H̄ 23 da. 21 da. p.o.					5	81 ± 7	5 ± 0.3	6 ± 0.4	0	0	atrophic
N 21 da.					3	224 ± 36	24 ± 0.6	8 ± 0.6	0	0*	sparse, few epithelioid
23 da					3	281 ± 22	25 ± 0.6	8 ± 1.3	0	0*	sparse, few epithelioid
42 da.					3	1284 ± 75	67 ± 8.5	26 ± 4.1	0	100 few	epithelioid
44 da.					3	1844 ± 105	78 ± 12	48 ± 3.9	0	100	epithelioid

* Spermatocytes abundant in all 21–23 da. normal rats, before and after injection.

Gonadotrophic potency of rat pituitaries in intact and hypophysectomized weanling males

It is evident from the response in the hypophysectomized rats that the testes of weanling rats were sufficiently mature to respond to the exogenous gonadotrophins. The lack of testicular response in intact weanlings might be due to the removal of some supporting pituitary hormone during purification of the sheep FSH and ICSH. On the other hand, the heterologous hormones might not be of equivalent value to homologous gonadotrophins. To test the action of rat pituitary hormones, castrate rat pituitaries were chosen due to their high gonadotrophin content. As shown in Table 6, extracts of such pituitaries (see appendix), given in unitages equivalent to effective doses of sheep gonadotrophins, gave results in both normal and hypophysectomized rats comparable to those just described for heterologous hormones. The normal development in intact recipients proceeded without interference, the only effect observed being acceleration in the growth of accessory organs. In the hypophysectomized recipients injected from time of operation, development was parallel in all respects to that observed in animals receiving comparable unitages of sheep gonadotrophins. The growth of testes and accessory organs was normal or in excess of normal; spermatids were present in all testes and sperm appeared in two-thirds of the rats at the precocious age of 44 days. The rat pituitary extracts injected contained all the pituitary hormones; therefore the homologous gonadotrophins, even though supplemented by non-gonadotrophins known to be synergic with gonadotrophins, were still ineffective in bringing about precocious development in the immature male.

Gonadotrophic potency of immature rat pituitaries

Homogenates of anterior pituitaries from immature male rats (23 to 33 days of age) were assayed for gonadotrophic content by injection for 15 days in the standard hypophysectomized 40 day old male rat. As shown in Table 6, the anterior pituitaries of these immature males contained adequate hormones to stimulate the reproductive system to full development for the chronological age (55 days).

Influence of FSH on maintenance and repair of the reproductive system of the hypophysectomized male rat by testosterone propionate

Provided it can be assumed that ICSH is effective in stimulation of testicular tubules through stimulation of Leydig cell secretion, and that FSH augments the action of ICSH, the question then arises whether FSH acts synergistically directly with ICSH or through the androgen secreted. Comparisons were accordingly made between the response to FSH combined with testosterone propionate and to FSH combined with ICSH. The

levels of testosterone propionate chosen were low but were known to be within the range effective in the 40-day-old hypophysectomized test animal (16). In maintenance studies the testosterone propionate was injected in daily doses of 10 or 50 μg alone and in combination with FSH, for periods of 15 days. The level of FSH injected, 2 R.U. daily, had only slight effects on testicular weight or differentiation and no effect on accessory organ weights. The lowest dose of testosterone propionate (10 μg), when administered alone from the time of hypophysectomy had but slight capacity to maintain testicular and accessory organ weights (Table 8). When it was injected with FSH a slightly greater degree of testicular weight maintenance resulted than from either component alone, and spermatids with compact nuclei remained adherent in some tubules. The combination did not significantly increase the weight of accessory organs resulting from testosterone propionate alone. Daily doses of 50 μg of testosterone given alone still caused only partial maintenance of testes but increased accessory organ weights above the normal 55-day range. When this level was combined with FSH, tubular differentiation advanced to formation of sperm at the normal age of 55 days, although testicular weights were only maintained at the 40-day level. FSH did not increase the capacity of testosterone to stimulate accessory organ development.

In attempts to repair the reproductive system after a 14 day regression period (Table 8), 10 μg daily doses of testosterone propionate did not increase the weight or differentiation in the atrophied testis even when the injection period was extended to 28 days, and caused only incomplete reinstatement of accessory organs. This dose of testosterone combined with 5 R.U. FSH (alone ineffective in repair) still resulted in slight or no repair of the testis and no further increase in accessory organ weights. The addition of growth and lactogenic hormones to this combination increased testicular weight and differentiation somewhat and caused a slight increase in accessory weights. Five times this dose of testosterone propionate (50 μg), a dose unable alone to repair the testis, did increase testicular weight when combined with FSH, although the combination did not significantly improve tubular development and did not further increase accessory organ weights. Testosterone injected in daily doses of 250 μg increased testicular weights above the condition at onset and a few spermatids were formed; at these daily doses the accessory organ weights were many-fold those in normal 55-day-old rats. The combination with FSH restored the weights of testes almost to the normal 40-day status, but what is even more important, accomplished spermatid and sperm formation. The accessory organs were far above 55-day normal range, and were somewhat larger than after testosterone alone, but as observed at lower dose levels, there was no definite evidence that FSH augmented the action of testosterone on accessory organs. This highest dose of testosterone propionate (250 μg). was

TABLE 8. MAINTENANCE AND REPAIR OF THE REPRODUCTIVE SYSTEM IN THE HYPOPHYSECTOMIZED MALE RAT BY TESTOSTERONE PROPIONATE ALONE AND COMBINED WITH FSH

Type of experiment	Daily dose		No. of rats	Organ weights (mg. ± standard error)			Testicular response* (percent of rats)		Adrenals (mg. ± S.E.)	B.W. change injection period (gm.)
	T.P. (μg)	FSH (R.U.)		Testes	Ventral prostate	Seminal vesicles	Sperm	Spermatids		
Maintenance										
40 da. at H & onset	10	—	4	564 ± 72	32 ± 2.8	30 ± 6.6	0	0	10 ±0.8	−20
Injection 15 da.	10	2	4	862 ± 76	45 ± 18	37 ± 6.3	50 few	50	9 ±0.6	−21
	50	—	5	770 ± 41	131 ± 14	389 ± 47	0	40 rare	16 ±3.5	− 8
	50	2	3	1334 ± 46	127 ± 14	282 ± 16	100 few	100	11 ±1.0	− 8
	—	2	18	708 ± 56	12 ± 0.6	14 ± 0.9	0	11	10 ±0.8	−20
Repair										
40 da. at H	10	—	5	276 ± 14	28 ± 5.5	51 ± 9.6	0	0	7 ±0.4	+ 5
Onset 14 da. p.o.	10	5	4	352 ± 21	28 ± 1.0	43 ± 3.9	0	0	8 ±0.2	+10
Injection 28 da.	10	5								
	+2.5 R.U. GH +2 I.U. Lact. H		5	552 ± 94	44 ± 2.2	73 ± 8.3	0	few, young	9 ±0.2	+47
	50	—	5	288 ± 22	174 ± 9.1	586 ± 48	0	0	9 ±1.1	+15
	50	5	5	414 ± 47	147 ± 5.5	586 ± 37	0	few, young	9 ±0.1	+17
	250	—	5	548 ± 70	397 ± 25	1552 ± 70	0	+ few	9 ±0.2	+19
	250	5	5	1032 ± 114	413 ± 41	1712 ± 69	60	+	8 ±0.6	+14
	—	5	5	286 ± 19	9 ± 0.9	10 ± 0.9	0	0	8 ±0.3	+ 5
Controls										
H 40 da., 14 da. p.o.			62	386 ± 19	11 ± 0.5	14 ± 0.6			9.4 ± 0.9	−21
40 da., 42 da. p.o.			2	214 ± 22	8 ± 0.3	11 ± 7.2	0	0	8.5 ± .65	− 5
N 40 da.			47	1372 ± 40	66 ± 2.5	34 ± 2.4	0	100		
55 da.			49	2448 ± 54	153 ± 6.7	250 ± 16	100	100		

* No Leydig cell maintenance or repair in any H rats.

low, compared with doses found effective by Nelson, and by Gaarenstroom and de Jongh for spermatogenesis in the regressed testis of hypophysectomized rats (3–6).

DISCUSSION

Although normal weanling male rats could not be matured precociously, they responded readily to pituitary gonadotrophins after hypophysectomy. Intact rats were likewise unresponsive to sheep pituitary gonadotrophins supplemented by other pituitary hormones known to be synergic with gonadotrophins, or to homologous gonadotrophic preparations containing these synergic substances. The pituitaries of such weanling males contained gonadotrophins in adequate amounts to cause sperm formation in hypophysectomized recipients. Some explanation must therefore be sought for the failure of precocious development in normal immature males, other than insufficiency of pituitary hormones, or lack of sensitivity of the testis to either heterologous or homologous pituitary hormones.

As reported here a daily dose of ICSH as low as $\frac{1}{4}$ R.U. maintained and advanced tubular development in the 40-day-old hypophysectomized rat. Such doses showed morphological evidence of partial interstitial cell maintenance but the accessory organs were not stimulated and much larger doses were required to maintain the pre-operative status and accelerate their growth. It can nevertheless be assumed that the androgen secreted by the stimulated Leydig cells was responsible for the development of the seminiferous epithelium. Higher doses of testosterone propionate are required for spermatogenic than for androgenic action. However, other synthetic androgenic steroids have been found to vary markedly in the ratio of their androgenic to spermatogenic potencies, some having higher gametogenic than androgenic properties (3, 17). The androgenic steroid secreted by the rat under the influence of ICSH may also excel in the capacity to stimulate tubules.

The similarity between the synergism resulting from FSH combined with testosterone propionate and that resulting from FSH combined with ICSH suggests that the mechanism of action in the two instances is the same, and that the FSH does not synergize directly with the ICSH, but rather with the androgen produced through the action of the ICSH.

An important role has frequently been attributed to FSH in control of the male reproductive system (6, 18–20) yet the exact role may be difficult to define. As pointed out, ICSH, administered immediately after hypophysectomy, had the capacity not only to stimulate Leydig cells and accelerate accessory organ growth but also to maintain and advance spermatogenesis and spermiogenesis. However, when injected after post-hypophysectomy atrophy of the testis, it did not act as a complete gonadotrophin though administered at higher doses and for twice the period needed for mainten-

ance. When it was supplemented by FSH, both repair and continued development of the testis and accessory organs followed, demonstrating that FSH does play a role in control of the male reproductive system. It is not easy, however, to determine just what contribution FSH makes in development of the testis. As shown here, the purest FSH preparations available had very limited capacity to maintain spermatogenesis, when given in the dose range showing no contamination with ICSH. Yet small doses (e.g., of 0.5 R.U.), which caused only slight or no acceleration of tubular activity, were synergic with ICSH. After a period of post-hypophysectomy regression FSH, which had scarcely detectable effect on the tubules, continued to act synergically with ICSH. The mechanism by which FSH furthers spermatogenesis and spermiogenesis therefore remains obscure.

It has been shown that growth and lactogenic hormones increased the action of ICSH, and increased further the action of combinations of FSH with ICSH, particularly on the accessory organs. It was surprising that two purified hormones caused such similar responses, qualitatively and quantitatively, more so considering that no dose-response relationship could be established between the amount of growth or lactogenic hormone injected and the extent of the augmentation. It was known that both hormonal supplements were contaminated with small amounts of other pituitary hormones: the contamination with gonadotrophins (FSH and ICSH) was so small in both the growth and lactogenic hormone preparations (see appendix) that it is improbable that they influenced the response; the other two main contaminants, TSH and ACTH, likewise did not appear to be responsible for the augmentation, since purified preparations of these hormones given alone or combined with gonadotrophins did not influence the testis or accessory organs. The augmentation of the gonadotrophic action of ICSH by growth and lactogenic hormones does not appear to be due to the contamination of either hormone by the other. The growth hormone at the 2.5 R.U. level had only slight lactogenic potency (0.01 I.U.). The Lact. H injected in males for the 15-day experimental period did not cause a significant increase in body weight or width of tibial epiphyseal cartilage. However, it may be noted that routine bioassay in hypophysectomized immature female rats of preparation NIH-P-S-3 disclosed some contamination with GH (0.03 R.U. in 1 I.U.). The other preparation of Lact. H used (L2378A), although also effective in augmentation, contained only about one third the GH contamination. More increase in accessory organ weights resulted from combinations in which the more contaminated preparation was used, so that some consideration must be given to the possible significance of contamination with GH. Perhaps one is inclined to attribute greater significance to the GH as an augmenting factor due to reports of beneficial effects of GH on the so-called "specific" action of several other hormones, such as, the increased calorigenesis when combined with TSH (13), increased mammary gland stimulation when combined

with Lact. H (21), increased male accessory organ response when combined with testosterone (22), and increased adrenal response when combined with ACTH (23). The participation of growth and lactogenic hormones in support of the male reproductive system has been reported previously by others though no agreement was reached as to which factor played the primary role and which was the contaminant (23–27).

It seems clear that in the male the ICSH is the primary anterior pituitary hormone responsible for the seminiferous epithelium as well as the Leydig tissue, inasmuch as no other pituitary hormone was effective in its absence. It is possible to evaluate the adequacy of ICSH in repair and maintenance of reproductive function quite accurately, inasmuch as one can give high doses without evidence of contaminating hormones (see appendix). ICSH has been purified so that six thousand times the minimal effective dose shows no contamination with the second gonadotrophin, FSH. This high degree of purification, considered with the very low doses of ICSH effective in the male, precludes the interpretation that the results of ICSH injection are attributable to the contaminating FSH. It is far more difficult, however, to investigate the role of FSH in the male since FSH has not been so completely freed from its capacity to stimulate intersititial tissue. Even when the most purified preparations of FSH are used one is restricted to those dose levels which do not show stimulation of the Leydig cells or androgen secretion, which judged by routine bioassay methods permits only a 40–70 fold range of doses. The sensitivity of the male to minute amounts of ICSH accentuates the difficulty inherent in this restricted range. It is particularly important therefore in evaluation of FSH preparations to bear in mind that ICSH exerts effects on testicular tubules at doses below those at which its other physiological attributes are shown.

APPENDIX

Biological Standardization of Hormones

Careful bioassays were made of all hormonal preparations, both for the specific hormonal potency and for contamination with other pituitary hormones. ICSH and FSH were assayed in hypophysectomized immature females of the Long-Evans strain by the method of Evans *et al.* (28, 29), as described again recently by Woods and Simpson (30). The rat unit (R.U.) of ICSH is defined as the minimal total dose, injected IP daily over a 3-day period, which initiates repair of the deficient interstitial cells in the hypophysectomized female rat. The R.U. of FSH is the minimal total dose among a series of graded doses injected SC once daily for 3 days which after 72 hours results in microscopically measurable follicular development with beginning antrum formation, an increase in follicular diameter from 375 μ to 450–500 μ, in two-thirds of the rats tested. (The effective dose for follicular development in 67% of the test animals, as determined graphically from the plot of the probit of percent response to the

log-dose (ED67), was found to correspond to the MED commonly determined by empirical observation (30).

For purposes of comparison, ICSH potency was also determined in hypophysectomized males by the ventral prostate method of Greep *et al.* (31), and comparisons of potency were made with NIH-LH-S1. The potency of FSH preparations as determined above was compared with that determined in the normal female by the augmentation test under the conditions described by Steelman and Pohley (32), and comparison of potency was made with NIH-FSH-S1.

Growth hormone was assayed by the tibia test (33, 34) and a unit (R.U.) was determined as the total dose in a graded series of dosages which produced 50 μ increase above the control width of the proximal epiphyseal cartilage of the tibia. Thyrotrophic activity (TSH) was determined histologically (35) ($\pm$designating a marginal response; +, epithelium higher cuboidal with less flattened, lighter staining nuclei and beginning resorption of colloid; ++, taller cuboidal epithelium with extensive resorption of colloid). TSH activity was also measured by radio-iodine uptake and was compared with the U.S.P. standard. Adrenocorticotrophic activity (ACTH) was determined histologically (36) in Sudan stained sections (+ indicated minimal response with lipid droplets less coarse than in hypophysectomized control; ++, subglomerular lipid free zone obliterated; +++, marked increase in width of cortex). ACTH activity was also measured by adrenal ascorbic acid depletion with comparison with the U.S.P. unit. Lact. H was determined in pigeons by the local crop sac method (37) and expressed in international units (I.U.).

Contamination with the specific target organ hormones was determined by injection of high doses in the immature hypophysectomized female rat, except in the case of Lact. H contamination which was determined by local crop injection of pigeons. Contamination of ICSH and ACTH with hypertensive principles was determined by the Endocrine Laboratories, Madison, Wisconsin, according to the method outlined in U.S. Pharmacopeia XV for vasopressin.

Hormonal Preparations Used; Source, Unitage, Degree of Purity

The ICSH preparations which were used in this study were prepared from extracts of sheep pituitary by fractionation with $(NH_4)_2SO_4$ followed by ion-exchange chromatography on DEAE-cellulose (38-40). Of the three ICSH preparations used, the MED in two of them (III-1A and III-58A) was 1.3 μg; the MED of the third (V-17A) was 1.0 μg. This potency is to be compared with MED of 5.0 μg for NIH-LH-S1, as determined by the same method. When assayed in the immature hypophysectomized male, III-58A in a total dose of 2.5 μg caused 100% increase in ventral prostate weight; an equivalent reaction resulted from 15 μg of NIH-LH-S1. Armour standard LH227-80 was equipotent with NIH-LH-S1 by both tests. Con-

tamination of ICSH preparations with FSH was less than 0.02% (i.e., they produced no follicular stimulation in hypophysectomized assay rats at 6.0 mg.). The contamination of ICSH with TSH was between 0.1 and 0.2% (MED TSH >500 <1000 μg), and with growth, lactogenic or adrenocorticotrophic hormones (injected in 0.9% NaCl, 4 day test) was less than 0.1% (MED >1000 μg). A preparation of ICSH containing 1000 R.U./mg. contained a contamination with vasopressor principles of less than 0.4 U.S.P. units per mg.

FSH was prepared from sheep pituitaries by the method described by Woods and Simpson (30). The four preprations used in this study had potencies and contamination with ICSH as follows:

Preparation No.	MED, FSH μg (H)	Multiple MED FSH containing MED ICSH (H)	Multiple of potency of NIH-FSH-S1 (N)
III-25C	4.3	22×	10×
III-72C	2.9	35×	18×
V-47C	2.3	33×	45×
IV-19F	1.7	70×	32×

These FSH preparations were contaminated with less than 0.1% of GH, Lact. H, TSH and ACTH (i.e., negative at 100 times the MED for FSH). The potencies of FSH standards donated by the Endocrinology Study Section, National Institutes of Health, and Armour were determined by the methods in use here in order to make comparisons with preparations used here: the MED for NIH-FSH-S1 in the hypophysectomized female rat was 55 μg; the MED for Armour Standard FSH 264-151-X was 110 μg. By the augmentation test in normal female rats NIH-FSH-S1 was 1.7 times as potent as Armour Standard FSH 264-151-X. NIH-FSH-S1 caused repair of interstitial cells in the hypophysectomized female rat at 5 times its MED for FSH. Armour Standard FSH 264-151-X showed ICSH contamination at 130 μg, approximately the MED for FSH.

Growth hormone (G-43) was prepared from sheep pituitaries by G. Noda according to the method of Ellis *et al.* (41) and had a 40 μg MED by the tibia assay. Contamination with lactogenic hormone was 0.1 I.U. per mg. Therefore, in the daily dose of 2.5 R.U., a level which was frequently injected in these studies, the equivalent of 0.01 I.U. lactogenic hormone per day was administered. Contamination with ICSH and TSH was detected at 2.5 mg., and for ACTH (in 0.9% NaCl, 4 day test) at 5.0 mg. Treatment of this GH preparation with periodate by the method of Geschwind and Li (42) reduced contaminants, increasing the MED for TSH and ICSH to 5.0 mg. and for ACTH to >5 mg., without affecting the growth promoting potency.

Two Lact. H preparations prepared from sheep pituitaries were used. NIH-P-S-3 was a purified preparation donated by the Endocrinology Study

Section, National Institutes of Health, stated to have a potency of 15 I.U. per mg. The MED for GH in this preparation was determined to be 2.5 mg.; the minimal effective dose for contamination with FSH, ICSH and TSH was >5 mg. (As stated by the Endocrinology Study Section, the contamination of the preparation, NIH-P-S-3, was TSH <0.0004 U.S.P. units per mg.; ACTH 0.00025 I.U. per mg. (by adrenal ascorbic acid depletion), vasopressin or oxytocin not more than 0.05 units per mg.; the GH content was stated to be >0.1 but <1.0% of the potency of a highly purified GH bovine (<0.01 units/mg.).) The second preparation of Lact. H used, L2378A, was donated by C. H. Li, and shown by standardization here to contain between 15 and 20 I.U. Lact. H per mg., and to have an MED for GH of 5.0 mg.

The TSH used (LLR-TSH-S1) was prepared from sheep pituitaries by L. L. Rosenberg by the method of Pierce *et al.* (43). The MED for initiation of thyroid repair in the hypophysectomized rat was 5 μg and by comparison with the U.S.P. standard, it contained 3.5–4.0 U.S.P. units/mg.

Sheep ACTH (LLR-110-D1) was prepared by L. L. Rosenberg by the method described by Rosenberg, Evans and Simpson (44). Standardization by the adrenal ascorbic acid depletion method showed the preparation to contain 100 U.S.P. units/mg.

For comparison with the gonadotrophic activity of purified sheep pituitary preparations, extracts were prepared of rat anterior pituitaries collected from normal and castrate adult males. The castrate males were 50 to 80 days old at operation and were sacrificed four to six weeks postoperative. Dissected anterior pituitaries were homogenized and extracted with 40% ethanol at 25° C, followed by precipitation in 80% ethanol at 3° C, then dialysis and lyophilization of the precipitate. The MED for FSH in the preparation from castrate pituitaries was 30 μg, for ICSH 5 μg, and for GH 40 μg. Anterior pituitaries were also collected from normal immature male rats (23 to 33 days old, body weight range 35 to 121 gms., average 57 gm.). These pituitaries were homogenized in 0.9% NaCl, and fresh homogenates were prepared for each experimental series and stored during the 4 day injection period at 5° C.

Crystalline testosterone propionate used in these studies was obtained from Mann Research Laboratories, Inc.

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AN UNIDENTIFIED I^{131} CONTAMINANT OF RADIOIODIDE PREPARATIONS[1,2]

ALVIN TAUROG

Department of Pharmacology, The University of Texas, Southwestern Medical School, Dallas, Texas

ABSTRACT

A non-iodide I^{131} component was observed in a number of samples of radioiodide obtained from E. R. Squibb & Sons during the period December 1959 through March 1960. This component, which is referred to as U, for unknown, is both biologically and chemically distinguishable from radioiodide. It resembles closely an I^{131} component, called Unknown 2, previously encountered in subcellular preparations incubated with radioiodide. U, which appears to be an oxidized form of inorganic iodine, is acid labile and is not as well utilized by thyroid tissue as is radioiodide. Formation of U in radioiodide preparations can be prevented by the presence of reducing agents such as sulfite, cysteine, or 1 methyl-2-mercaptoimidazole. The unknown I^{131} component was not observed in I^{131} samples obtained from Oak Ridge National Laboratory, nor was it present in more recent samples of I^{131} obtained from E. R. Squibb & Sons in which special precautions were taken to prevent its formation.

DURING the course of experiments in which thyroid tissue slices were incubated in a buffer medium containing added radioiodide obtained from E. R. Squibb & Sons (Squibb Iodotope, oral solution), I^{131} uptakes by the tissue were only about one-third as great as those that had been observed in many previous experiments employing I^{131} obtained from Oak Ridge National Laboratory. The low uptakes were subsequently found to be due to the presence of an extraneous I^{131} component in the Squibb radioiodide preparation. This component, which comprised as much as 50 per cent of the total I^{131} in some samples, is referred to in this communication as U, for Unknown. Its chromatographic and chemical properties were found to be very similar to those of another unidentified I^{131} component, called U-2, which Taurog *et al.* encountered in thyroid and submaxillary subcellular preparations incubated with I^{131} (1). The present communication deals with the chemical and biological properties of U and with its relationship to unidentified I^{131} components previously reported by other workers (2, 3) in radioiodide preparations.

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[1] Aided by Grant No. A-3612 from the U. S. Public Health Service.

[2] A preliminary account of this work was presented before the Endocrine Society (Program of the Forty-Second Meeting, Miami Beach, Florida, June 9–11, 1960, p. 49).

EXPERIMENTAL

Tissue Slice Experiments

Thyroid tissue was collected from freshly slaughtered animals at a local abattoir and brought to the laboratory packed in ice. Slices of the trimmed glands were prepared manually with a razor blade and collected in a shallow dish of Krebs-Ringer-bicarbonate buffer kept on ice. When possible, slices from a single gland were used for all the samples in one experiment to avoid differences in activity between glands of different animals. The slices were blotted on filter paper immediately before weighing, and 200 mg. portions were added to 3 ml. of Krebs-Ringer-bicarbonate buffer containing about 0.2 μc. of radioiodide. Incubations were made in 25 ml. glass-stoppered flasks, which were flushed with a mixture of 95 percent O_2 and 5 percent CO_2 immediately before being placed on the shaking incubator. After 90 to 120 minutes of incubation at 37–38° C, the contents of each flask were poured through a coarse filter, and the slices were picked up individually with forceps, quickly dipped several times in cold saline or buffer, and placed directly in a counting vial for I^{131} assay.

In Vivo *Experiments*

Sprague-Dawley male rats were purchased from Holtzman Animal Farms and were maintained on a commercial diet.[3] The mean body weights in different experiments varied from 150 to 250 grams. Carrier-free I^{131} (0.5–1 μc.) was administered orally by gastric intubation, intraperitoneally, or intravenously via tail vein. Thyroids were removed after 20–24 hours and counted in the scintillation well-counter.

Radioiodide Preparations

Comparisons were made between Squibb Iodotope, oral solution, referred to hereafter as Squibb Iodotope, and I^{131} purchased directly from Oak Ridge National Laboratory and referred to hereafter as Oak Ridge I^{131}. In one experiment observations were also made with Abbott Oriodide, oral solution, obtained from Abbott Radio-Pharmaceuticals, Oak Ridge, Tennessee. All I^{131} solutions were received in the carrier-free form. Iodide carrier was added in some experiments, as indicated below.

Chromatography

Ascending filter paper chromatography of the I^{131} solutions was carried out on strips of Whatman 3 MM paper, 10×35.5 cm. The solvent employed in most instances was butanol-ethanol-2N NH_4OH (5:1:2). Other details of the chromatographic technique were as previously described (4). In all instances, unless otherwise stated, 1-methyl-2-mercaptoimidazole (Tapazole,[4] 7×10^{-4}M) and iodide (5×10^{-5}M) were added just before chromatography to the solution to be chromatographed to prevent artifacts of chromatography.

Elution

The section of the chromatogram corresponding to the U band was located by radioautography and cut out in the form of a narrow, rectangular strip, approximately 1.5×10 cm. The strip was pointed at one end, and the opposite end was wetted to a distance of about 1 cm. with 0.15 M $NaHCO_3$. The wetted end was attached by simple adhesion to a wetted filter paper wick held in place in a glass trough partially filled with 0.15 M $NaHCO_3$. Ten or twelve strips were so treated and simultaneously eluted under a bell jar at room temperature, the eluate being collected in small vials. The I^{131} in the U band

[3] Uncle Johnny's BSD, Uncle Johnny Mills, Houston, Texas.

[4] Kindly supplied by Eli Lilly & Company, Indianapolis, Indiana.

was quantitatively eluted from the paper when approximately 0.3 ml. of $NaHCO_3$ solution had collected in the vial.

RESULTS

I^{131} Uptakes in Thyroid Tissue Slices

Results of several experiments in which both Squibb Iodotope and Oak Ridge I^{131} were employed are shown in Table 1. Use of the Squibb prepara-

TABLE 1. I^{131} UPTAKES IN THYROID TISSUE SLICES INCUBATED WITH SQUIBB IODOTOPE-ORAL SOLUTION OR OAK RIDGE I^{131}

Animal	I^{131} Source	Age of I^{131} from date received	Carrier iodide added	Percent uptake of added I^{131} in slices
		(days)	(μg per cent)	
Hog	Squibb	3	0	25–27
	Oak Ridge	0	0	63–71
Hog	Squibb	0	0	27–34
	Squibb	8	0	17–18
	Oak Ridge	5	0	57–66
Hog	Squibb	6	7	17–18
	Oak Ridge	48	7	58–63
Cow	Squibb	16	7	25–26
	Oak Ridge	37	7	83–89

tion consistently led to uptakes only one-half to one-third as great as those observed when the Oak Ridge I^{131} was employed. The presence or absence of added iodide carrier did not affect the results, nor did the age of the sample from the time it was received appear to have any effect.

Thyroid I^{131} Uptakes In Vivo

When rats were injected intraperitoneally with Squibb Iodotope, thyroid I^{131} uptakes were significantly less than those observed following similar

TABLE 2. THYROID I^{131} UPTAKES IN RATS AFTER ORAL OR INTRAPERITONEAL ADMINISTRATION OF SQUIBB OR OAK RIDGE I^{131}

I^{131} Source	Route of administration	No. of rats	Interval	Percent uptake in thyroids
			(hours)	
Squibb	Oral	8	21	12.6 ± 1.9*
Squibb	I.P.	7	21	2.7 ± 1.2
Oak Ridge	Oral	7	21	10.3 ± 1.9
Oak Ridge	I.P.	7	21	18.3 ± 2.8
Squibb	Oral	5	20	7.0 ± .13
Squibb	I.P.	5	20	2.4 ± .10
Squibb	Oral	4	18	7.0 ± .62
Squibb	I.P.	4	18	3.7 ± .77
Oak Ridge	Oral	4	18	9.6 ± .93
Oak Ridge	I.P.	4	18	9.9 ± .66
Squibb	Oral	5	20	9.6 ± 1.3
Squibb	I.P.	5	20	4.8 ± .56

* Mean ± standard error.

injection of Oak Ridge I^{131} (Table 2). This difference, however, was largely abolished if the I^{131} was administered by gastric intubation. The latter mode of administration gave much higher uptakes than the intraperitoneal route when the Squibb prepration was employed, increasing the values to the range observed after administration of Oak Ridge I^{131}. Route of administration did not have any consistent effect on the results obtained with Oak Ridge I^{131}.

Chromatography of Squibb and Oak Ridge I^{131}

Figure 1 illustrates the chromatographic purity of one pair of Oak Ridge and Squibb I^{131} preparations employed in the above studies. It is apparent that the Squibb product contained a major I^{131} contaminant, referred to here as U, and several minor contaminants. The Oak Ridge sample, on the other hand, contained very little non-iodide I^{131}.

During the course of this investigation, chromatograms similar to that

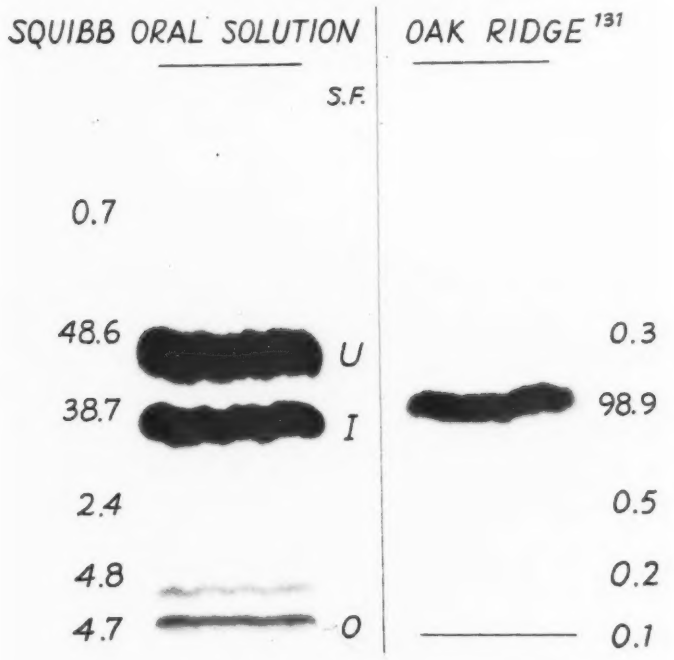

FIG. 1. Radioautographs of chromatograms of Squibb Iodotope (left) and Oak Ridge I^{131} (right), chromatographed four and five days, respectively, after the I^{131} solutions had been received. Tapazole (75 μg/ml.) and carrier iodide (5 μg/ml.) were added before chromatography. The numbers refer to the percentage of I^{131} on the chromatogram in the various bands.

Symbols: O, origin; I, iodide; U, unknown; S.F., solvent front.

Chromatographing solvent: Butanol-ethanol-2N NH_4OH, 5:1:2.

shown in Figure 1 were obtained with eleven different Squibb Iodotope preparation (from December, 1959 through March, 1960), whereas six different Oak Ridge radioiodide samples tested over the same period showed negligible contamination.

The use of several different chromatographing solvents to illustrate the presence of the U contaminant in Squibb Iodotope is shown in Figure 2.

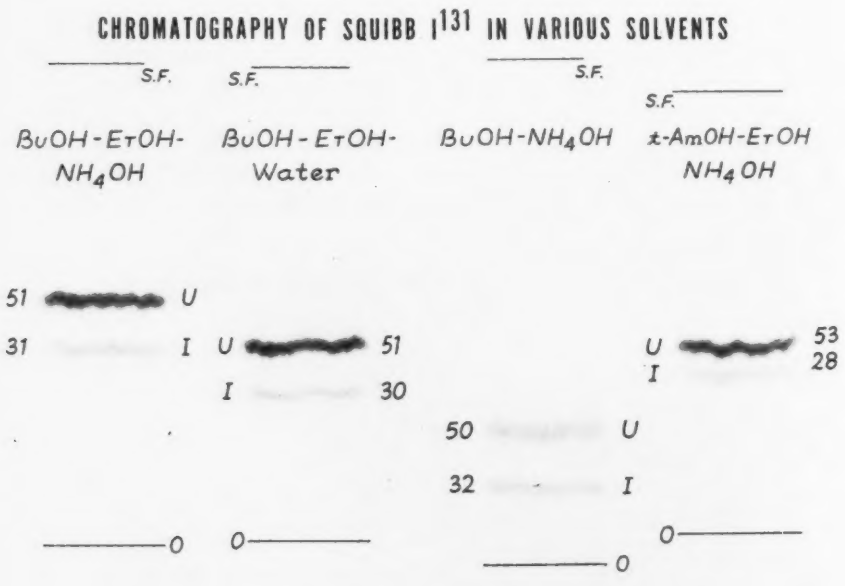

FIG. 2. Radioautographs of chromatograms of Squibb Iodotope in different solvent systems. The compositions of the solvents were as follows: Butanol-ethanol-2N NH_4OH, 5:2:1; butanol-ethanol-water, 5:1:2; butanol-2N NH_4OH, 10:1; t-amyl alcohol-ethanol-2N NH_4OH, 5:1:2. Tapazole (75 μg/ml.) and carrier iodide (5 μg/ml.) were added before chromatography. Numbers and symbols have the same meaning as in Figure 1.

There is good agreement among the different solvents for the percent distribution of the I^{131}.

Formation of U from Oak Ridge I^{131}

Discussion with Dr. Howard Stern of E. R. Squibb and Sons revealed that Squibb Iodotope is prepared from Oak Ridge or Canadian Atomic Energy I^{131} by dilution with .9% saline, adjustment of pH toward neutrality with HCl, and addition of .9% benzyl alcohol. These conditions were partially reproduced in this laboratory by adding 100 μl. of Oak Ridge I^{131} to 0.5 ml. of .15M saline containing 5 μl. of benzyl alcohol. Immediately after mixing, 25 μl. of the solution was withdrawn for chromatography.

The solution was then placed in a lead shield at room temperature, and 25 μl. aliquots were withdrawn for chromatography at intervals thereafter. The chromatography was always performed in the presence of Tapazole (approximately 10^{-3}M) and carrier iodide (approximately 10^{-4}M). In most experiments, there appeared on the chromatograms within the first day or two after storage an I^{131} component which resembled in all respects the unknown observed in the Squibb preparations. This component increased with time and in one experiment (illustrated in Figure 3) it represented over 50 percent of the total I^{131} on the 27th day after initial mixing. Appearance of this component was greatly inhibited, however, in two other mixtures of saline, benzyl alcohol, and Oak Ridge I^{131} to which Tapazole (5×10^{-4}M) or iodide (3×10^{-5}M) had been added immediately after mixing so that these agents were present throughout the period of standing (Fig. 3).

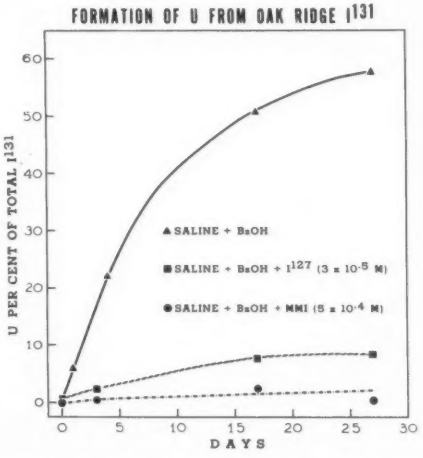

FIG. 3. Formation of U with time in mixture of 0.15 M saline, 0.9% benzyl alcohol, and Oak Ridge I^{131}. See text for details.

Considerable variation in the amount of U formation was noted in different experiments. In two experiments, I^{131} plus saline, but without benzyl alcohol, gave rise to appreciable U formation after 10 to 20 days of standing. However, in all instances, the presence of a small concentration of Tapazole or of carrier iodide completely inhibited or greatly reduced the formation of U.

Inhibition of U formation by the presence of a small concentration of Tapazole suggested that its formation involves an oxidative step, since Tapazole is known to block oxidation of iodide. The results with iodide carrier suggested that the formation of U in I^{131} preparations is a tracer effect related to the minute concentration of iodide in carrier-free I^{131} preparations. It seems likely, therefore, that U formation from carrier-free I^{131} may be related to minute concentrations of oxidizing impurities in the water and in the reagents used for admixture. The role of I^{131} radiation itself in the formation of U was not investigated, but it may be

pointed out that the I^{131} concentrations (mc./ml.) of Oak Ridge I^{131} samples which showed no extraneous bands were 20–40 times greater than those of Squibb radioiodide samples.

Instability of U in Acid

As indicated above, the U band observed in Squibb Iodotope is very similar in Rf value to an unidentified I^{131} band which Taurog *et al.* described in 1957 in thyroid and submaxillary subcellular preparations incubated with Oak Ridge radioiodide (1). The latter unidentified component was designated as Unknown 2, or U-2. An interesting characteristic of U-2 was its rapid conversion to iodide when exposed to acid. Similarly, the U band observed in the Squibb radioiodide was also acid labile. If a sample of Squibb radioiodide previously shown to contain a high percentage of U was acidified with HCl, then neutralized with $NaHCO_3$ immediately before chromatography, only a band for iodide I^{131} was observed on the radioautogram. This suggested an identity between the U band of the present study and the U-2 band of the previous study. It also offers an explanation for the discrepancy between oral and intraperitoneal uptakes observed after administration of Squibb Iodotope, since it would be expected that the unknown would be rapidly converted to iodide I^{131} in the acid of the stomach.

Formation of U in the Absence of Organic Material

The U-2 band previously described (1) was thought to be an organic I^{131} compound, and it was suggested that it might contain a labile N-I bond. However, it seemed very unlikely that the U band of the present investigation was organic in nature, since the only organic material present in solutions in which it appeared was benzyl alcohol. To obtain further information on this point, Oak Ridge I^{131} was mixed with alkaline hydrogen perioxide in the absence of any organic material. Iodide carrier was added in most experiments to avoid trace effects. Samples were withdrawn for chromatography after approximately two hours, and a band that appeared to be identical with the U band of the Squibb Iodotope was observed on all the chromatograms. The reaction was successful only when high concentrations of the H_2O_2 were employed. Results of some typical experiments illustrating the conditions and percentage yields are recorded in Table 3.

Experiments with Eluates of U

Chromatograms containing an appreciable percentage of their I^{131} in the U band were prepared from mixtures of saline, benzyl alcohol, and Oak Ridge I^{131} and also from treatment of Oak Ridge I^{131} with alkaline H_2O_2. The I^{131} in the U band was eluted from the paper with a small volume of 0.15 M $NaHCO_3$, as described above. Eluates from several paper chromato-

TABLE 3. FORMATION OF U FROM ALKALINE H_2O_2 AND IODIDE
(Chromatographed 100–150 minutes after mixing)

Initial concentration of				Percent of I^{131} on chromatogram in		
H_2O_2	Iodide	NH_4OH	NaOH	U	I	Other
Mols/Liter						
7	1×10^{-4}	2		34	62	4
7	1×10^{-4}		2×10^{-3}	37	59	4
7	1×10^{-3}	2		31	63	6
7	1×10^{-3}		2×10^{-3}	39	54	6
1.5	1×10^{-4}	2		13	85	2
1.5	1×10^{-4}		2×10^{-3}	23	75	2
7	0		1×10^{-3}	26	35	39
3	0		1×10^{-3}	14	68	18
1	0		2×10^{-3}	1.6	98	0

grams were pooled and the following experiments performed:

Acid lability—Aliquots of the U eluate were treated with acid, alkali, and a range of buffers to provide final pH values extending from 2.3 to eleven. After standing for 15 to 60 minutes, those solutions which were under pH 7 were made slightly alkaline, and the samples were all rechromatographed. A portion of the untreated U eluate was always chromatographed for comparison, since 5–15% iodide I^{131} was present even without any treatment. Exposure of the U eluate to mild acidity resulted in reduction or complete disappearance of the U band. U elates prepared from saline-benzyl alcohol-I^{131} mistures yielded the acid lability curve shown in Figure 4, indicating complete disappearance of the U band below pH five. This curve is almost identical with that reported for U-2 in the previous study (1). However, U elates prepared from mixtures of I^{131} and alkaline H_2O_2 usually showed somewhat greater resistance to acid, especially if carrier iodide had been added. In the latter case, approximately 25% of the initial U still remained after exposure to pH 2.5 for several hours at 37° C. The reasons for this difference in behavior of U eluates prepared

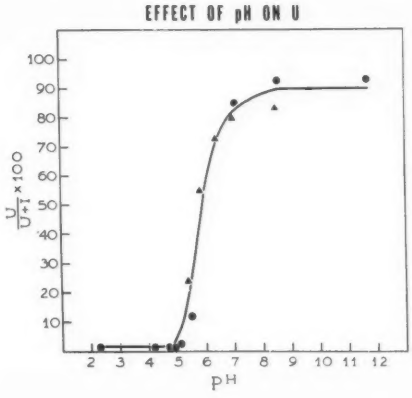

FIG. 4. Effect of pH on U eluate. See text for details.

TABLE 4. THYROID I^{131} UPTAKES *In Vivo* AND *In Vitro* WITH ELUTED U

Exp. No.	I^{131} component	Source of I^{131}	Thyroid I^{131} uptake in rats			I^{131} uptake in thyroid slices
			Interval (hours)	Route of administration		
				Oral	I.P.	I.V.	
1	Eluate of U	Chromatogram of saline-BzOH-I^{131}	6	3.9 ± .3 (5)†	2.7 ± .2 (5)†		11–13
	Radioiodide	Oak Ridge	6	4.3 ± .2 (5)	5.3 ± .3 (5)		46–56
2	Eluate of U	Chromatogram of saline-BzOH-I^{131}	23	8.2 ± .8 (4)	3.3 ± .4 (4)		21–22
	Radioiodide	Oak Ridge	23	10.4 ± .6 (4)	9.1 ± .8 (4)		66–69
	Eluate of I	Chromatogram of saline-BzOH-I^{131}	23	6.7 ± .8 (4)	6.6 ± .6 (4)		53–58
	Eluate of U (acidified)	Chromatogram of saline-BzOH-I^{131}	23				65–70
3	Eluate of U	Chromatogram of alkaline $H_2O_2+I^{131}$	23	7.9 ± 1.1 (5)		4.0 ± .7 (5)†	
	Radioiodide	Oak Ridge	23			9.0 ± 1.1 (5)	
4*	Eluate of U	Chromatogram of alkaline $H_2O_2+I^{131}$	22	15.4 ± .3 (4)	9.7 ± .2 (3)		
	Eluate of U (acidified)	Chromatogram of alkaline $H_2O_2+I^{131}$	22	21.3 ± 2.9 (4)	18.5 ± 2.7 (4)		
	Eluate of I	Chromatogram of Oak Ridge I^{131}	22	16.5 ± 1.9 (4)	12.4 ± .7 (4)		
	Radioiodide	Oak Ridge	22	19.0 ± 1.6 (4)	21.4 ± 1.9 (4)		

* These rats were on a low I diet.
† The numbers in parentheses indicate the number of rats in each experiment.

by the two methods are not apparent. However, since in other tests the U eluates prepared by the two methods proved indistinguishable, it is assumed here that the same unknown is produced by both procedures.

I^{131} uptakes in vivo *and* in vitro—Table 4 summarizes uptake studies in which U eluates were used as a source of I^{131}. It is evident from the *in vitro* studies that I^{131} in the form of U is not nearly as readily taken up by thyroid tissue as is I^{131} in the form of iodide. The *in vivo* experiments demonstrate that thyroid I^{131} uptakes were considerably higher after oral administration than after intraperitoneal or intravenous injection of U eluate, findings similar to those observed after administration of Squibb Iodotope. Of particular interest are the observations shown in Experiments 2 and 4 that previous acidification of the U eluate led to I^{131} uptakes comparable to those observed with Oak Ridge I^{131}. This confirms the view that treatment of U with acid converts all of its I^{131} to iodide. The I eluates referred to in Table 4, on rechromatography, showed an appreciable part of their I^{131} in a band similar to U. In experiment 4, for example, the I eluate showed 18 percent of its total I^{131} in this form and an additional 25 percent in other non-iodide forms. Presumably, conversion of I to U occurs on the paper chromatogram through air oxidation during the drying period. The presence of extraneous I^{131} in the I eluate probably accounts for the low I^{131} uptakes obtained with the preparation.

Electrophoresis—As illustrated in Figure 5, electrophoresis of U eluate showed U to be an anion with less than half the mobility of iodide. This was true both at pH 7.0 and at pH 8.5. The pattern shown in Figure 5 is very similar to that published previously (1) for U-2, supporting the view that U of this study and U-2 of the previous study are identical.

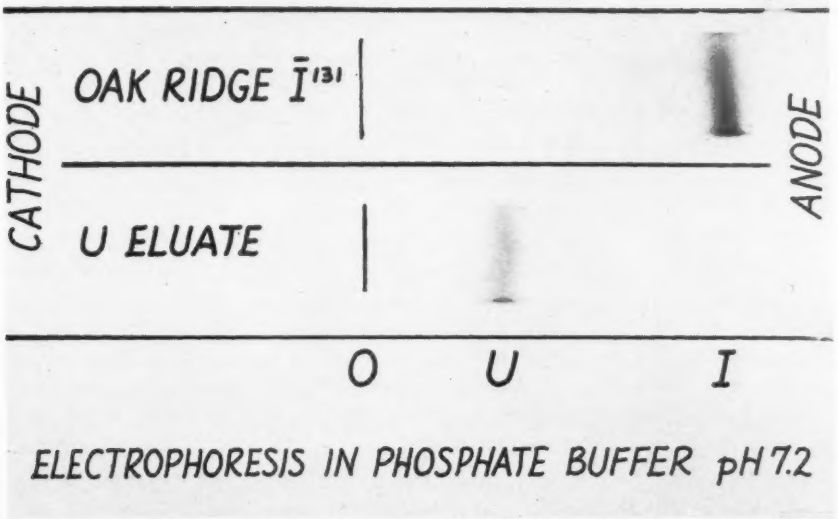

FIG. 5. Radioautograph of electrophoretigram of U eluate and Oak Ridge I^{131}. Electrophoresis was carried out in a Spinco-Durrum cell, 5.5 mA for 75 minutes.

Effects of heat, iodide carrier, reducing agents, and versene (Table 5)—When U eluates in $NaHCO_3$ solution were heated at 100° for one hour and rechromatographed, most of the I^{131} appeared as iodide on the chromatogram. U eluate in $NaHCO_3$ did not exchange readily with carrier iodide, nor was it appreciably reduced by 2×10^{-3}M thiosulfate or sulfite, or by 0.01M cysteine. However, in phosphate buffer at pH 7.0, these

TABLE 5. EFFECT OF VARIOUS AGENTS ON U ELUATE

Source of U eluate	Treatment of U eluate		Medium and pH	Percent of initial U remaining
Chromatogram of mixture of alkaline H_2O_2 and I^{131}	2×10^{-3}M $Na_2S_2O_3$	1 hr., room temp.	Bicarbonate, pH 8.6	97
Chromatogram of mixture of alkaline H_2O_2 and I^{131}	2×10^{-3}M $Na_2S_2O_3$	1 hr., room temp.	Phosphate, pH 7.0	52
Chromatogram of mixture of alkaline H_2O_2 and I^{131}	2×10^{-3}M Na_2SO_3	1 hr., room temp.	Bicarbonate, pH 8.6	99
Chromatogram of mixture of alkaline H_2O_2 and I^{131}	2×10^{-3}M Na_2SO_3	1 hr., room temp.	Phosphate, pH 7.0	56
Chromatogram of mixture of alkaline H_2O_2 and I^{131}	.01M cysteine	1 hr., room temp.	Bicarbonate, pH 8.6	97
Chromatogram of mixture of alkaline H_2O_2 and I^{131}	.01M cysteine	1 hr., room temp.	Phosphate, pH 7.0	52
Chromatogram of mixture of alkaline H_2O_2 and I^{131}	10^{-3}M iodide	6 hr., 37 degrees	Bicarbonate, pH 8.6	93
Chromatogram of mixture of alkaline H_2O_2 and I^{131}	10^{-2}M iodide	6 hr., 37 degrees	Phosphate, pH 7.0	39
Chromatogram of mixture of alkaline H_2O_2 and I^{131}	10^{-2}M EDTA*	2 hr., room temp.	Bicarbonate, pH 8.6	100
Chromatogram of mixture of alkaline H_2O_2 and I^{131}	10^{-2}M Tapazole	2 hr., room temp.	Bicarbonate, pH 8.6	99
Chromatogram of mixture of alkaline H_2O_2 and I^{131}	Heated to 100°	75 min.	Bicarbonate, pH 8.6	30
Chromatogram of mixture of saline-BzOH-I^{131}	10^{-2}M iodide	1 hr., room temp.	Bicarbonate, pH 8.6	100
Chromatogram of mixture of saline-BzOH-I^{131}	10^{-2}M iodide	1 hr., room temp.	Bicarbonate, pH 8.6	80
Chromatogram of mixture of saline-BzOH-I^{131}	10^{-3}M EDTA	1 hr., room temp.	Bicarbonate, pH 8.6	99
Chromatogram of mixture of saline-BzOH-I^{131}	10^{-2}M EDTA	1 hr., room temp.	Bicarbonate, pH 8.6	93

* Ethylenediamine tetraacetic acid, Na salt.

reagents did affect U eluate as indicated in Table 5. Versene (ethylenediamine tetra-acetic acid, Na salt), even at a concentration of 0.01M, produced no change in U in $NaHCO_3$ solution.

DISCUSSION

In 1959, Doctor and Trunnell (2) reported that solutions of Na I^{131}, obtained from either Abbott Radio-Pharmaceuticals or from E. R. Squibb & Sons, developed several extraneous I^{131} bands upon storage in the laboratory at room temperature. This conclusion was based on chromatography of three separate batches of Na I^{131} which were one, four, and seven weeks old. The older samples contained the most extraneous I^{131}, although change with time in any particular shipment was not reported, and the evidence was not conclusive that the differences observed were actually due to the length of storage in the laboratory. However, there was little question from these results that extraneous bands can appear on chromatograms of radioiodide, and these can in fact comprise the greatest part of the total I^{131}.

Pitt-Rivers and Wolff (5), in a brief comment on the paper of Doctor and Trunnell, suggested that the results obtained by the latter workers could be attributed to the fact that the radioiodide used in all their experiments was carrier-free. They stated that addition of a small amount of carrier iodide to radioiodide solutions, either fresh or old, before chromatography, results in the elimination of extraneous bands, and they concluded that the results obtained by Doctor and Trunnell were chromatographic artifacts.

The findings reported in the present investigation support the conclusion of Doctor and Trunnell that commercial radioiodide solutions may contain major I^{131} contaminants. The unidentified contaminant observed here in Squibb Iodotope, oral solution, was not eliminated by addition of iodide carrier or by addition of Tapazole. Ample evidence is presented to indicate that it is both chemically and biologically different from iodide, and that it does not arise simply as a chromatographic artifact.

The I^{131} contaminant did not appear in several different Oak Ridge I^{131} preparations, presumably because the concentration of reducing agent (sulfite) in this preparation is high enough to prevent oxidation of iodide. It may be postulated that the appearance of the contaminant in the Squibb preparation was due to dilution of the reducing agent, since the preparation of Squibb Iodotope entails dilution of an initial Oak Ridge or Canadian Atomic Energy radioiodide sample. Indeed, more recently, Squibb Iodotope is commercially prepared with the addition of cysteine as reducing agent, and the product no longer shows the presence of the U contaminant. It also yields I^{131} uptakes both *in vivo* and *in vitro* comparable to those obtained with Oak Ridge I^{131}. Similar results were obtained with one sample of Abbott Oriodide, oral solution (obtained from Abbott Radio-Pharmaceuticals), which also contained added cysteine.

There is little doubt that the U described in this communication is identical with, or very closely related to, the U-2 described previously (1). Although it was postulated previously that U-2 was not an inorganic form of iodine, this view must be revised on the basis of the present data. The original proposal was made primarily because no known form of inorganic iodine could explain the properties observed for U-2. However, since an I^{131} component which appears to be identical with U-2 and also with the unknown component in Squibb Iodotope can be formed by treatment of radioiodide with alkaline H_2O_2 in the absence of any organic material, it must be concluded that the unknown is some oxidation form of inorganic iodine.

The electrophoresis results indicate that U cannot be a cationic form of iodine, such as I^+. Moreover, the behavior of labeled U on electrophoresis and chromatography did not correspond to results obtained with known I^{131}-labeled preparations of elemental iodine (I_2), triiodide (I_3^-), iodate (IO_3^-), or hypoiodite (IO^-). The possibility was considered that U might be a polyoxyanion of iodine (not, however, IO_3^- or IO^-) which, on acidification, reacted with iodide to form I_2. In this connection, it should be pointed out that neither the chromatographic nor the electrophoretic procedure used here successfully separated iodide-I^{131} from I_2^{131}. However, since the I^{131} of the U eluate could not, after acidification, be extracted with chloroform, it was not considered likely that I_2^{131} had been formed. Further experiments are necessary to establish the exact nature of U.

Doctor (3) reported biological results with the major I^{131} contaminant which he encountered in commercial radioiodide preparations. This component, which he referred to as the "extraneous band," is very likely identical with the U of the present investigation. Injection of eluates of the extraneous band led to thyroidal I^{131} uptakes in chicks only about one-third as great as those observed following injection of chromatographically pure iodide I^{131}. The results reported here with intact rat thyroids and with hog and cow thyroid slices are in agreement with the findings of Doctor.

On the basis of the results obtained here, it would be expected that the oral use of radioiodide preparations containing appreciable percentages of U would lead to reduced thyroid I^{131} uptakes in patients with achlorhydria, and perhaps even in those with markedly reduced stomach acidity for any reason, as, for example, after partial gastrectomy. The absence of food from the stomach did not reduce thyroid I^{131} uptakes in rats after oral administration of Squibb Iodotope containing a high percentage of U, even though under these conditions the time of retention in the stomach was very likely reduced. Probably conversion of U to I^{131} was rapid enough at the pH and temperature inside the normal stomach so that time of exposure to stomach acid was not an important factor.

After completion of the present manuscript, a paper by DeGroot and Berger (6) appeared in which they also concluded that U-2 of Taurog

et al. is an inorganic oxidation product of iodide. Their findings with regard to the formation and chemical characteristics of U-2 are very much in accord with those reported here.

Addendum

While the present manuscript was in press, Ahn and Rosenberg (7) described an unknown I^{131} component, "S," encountered in commercial radioiodide preparations. Their unknown "S" is identical in almost all respects to the U described here.

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THE INTERSTITIAL CELL STIMULATING ACTIVITY OF OVINE, EQUINE AND HUMAN LUTEINIZING HORMONE PREPARATIONS IN THE HYPOPHYSECTOMIZED MALE RHESUS MONKEY[1]

E. KNOBIL[2] AND J. B. JOSIMOVICH[3]

Department of Physiology, Harvard Medical School, Boston, Massachusetts

ABSTRACT

Luteinizing hormone preparations of non-primate (sheep and horse) as well as of primate origin (human pituitary and placental) were found to stimulate the interstitial cells of Leydig in hypophysectomized male rhesus monkeys. Although quantitative differences in the efficacy of the various LH preparations were noted, it is concluded that an absolute "species specificity" as described for the action of growth hormone in primates is not attributable to the interstitial cell stimulating activity of luteinizing hormone.

ATTEMPTS to produce ovulation in suitably prepared hypophysectomized monkeys by the administration of non-primate luteinizing hormone (LH) preparations have been unsuccessful to date (1). Since the experimental induction of ovulation in such animals was achieved with human chorionic gonadotropin (HCG), albeit with some difficulty, the question arose whether the apparent inefficacy of the non-primate preparations was due to "species specificity" as demonstrated for growth hormone (2) or whether it was attributable to inadequacies in the mode of their administration.

In intact, immature and adult, female rhesus monkeys Simpson and van Wagenen (3) found that while ovulation occasionally followed injection of sheep pituitary preparations, it was produced with remarkable regularity by the administration of monkey pituitary extracts. The role of endogenous gonadotropin secretion in the intact animals responding to sheep hormone could not be assessed, however, in these elegant experiments.

Because the use of ovulation in the hypophysectomized monkey as an index of LH activity presents considerable experimental difficulties, it was felt that the response of the hypophysectomized male to various LH preparations would provide a simpler system for the investigation of the prob-

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[2] Present address: Department of Physiology, University of Pittsburgh, School of Medicine, Pittsburgh 13, Pennsylvania.

[3] Josiah Macy, Jr. Foundation Research Fellow. Present address: Boston Lying-In Hospital, Boston 15, Massachusetts.

lem since manifestations of "species specificity" would lead to conclusions applicable to both sexes.

While a few reports dealing with the action of LH preparations in the intact male rhesus monkey have appeared (4, 5, 6), definitive information regarding the effectiveness of such preparations in the hypophysectomized male rhesus monkey is not available.

The following study was undertaken to determine whether LH preparations of sub-primate origin possess interstitial cell stimulating activity when administered to hypophysectomized male monkeys using alterations in the appearance of the secretory epithelium of the seminal vesicle as the principal criterion of androgen production (7).

MATERIALS AND METHODS

Nine hypophysectomized and two normal mature male rhesus monkeys were studied. The animals were hypophysectomized by the parapharyngeal route while still sexually immature, 3½ months to 4 years prior to the experiments. Completeness of hypophysectomy was evidenced by increased insulin sensitivity, cessation of growth and sexual development and by decreased thyroid function. Gross euthyroidism was induced, when necessary, with desiccated thyroid (2). Control wedge biopsies of the testes and of the seminal vesicles were obtained at laparotomy under pentobarbital anesthesia. A portion of the testicular biopsy specimen was fixed in 10% formalin, embedded in 25% gelatin, sectioned at 10 μ on a freezing microtome and stained with Sudan black B. The remainder was fixed in Bouin's solution, embedded in paraffin, sectioned at 5μ and stained with hematoxylin and eosin. Sections of formalin-fixed seminal vesicle were similarly prepared for hematoxlyin and eosin staining.

Hormone administration was begun one to three weeks following the control biopsy. All preparations were administered subcutaneously in 1 ml. of 10–12% gelatin. The injections were continued for 14 days and a biopsy of the contralateral testis and seminal vesicle was performed on the day following the last injection and the tissues prepared as before. Details of the animals, hormone preparations and dosages used are given in Table 1.

TABLE 1

Monkey	Wgt. (kg.)	Months after hypophysectomy	Preparation used	Dose[a]
166	6.4	5	Human pituitary gonadotropin concentrate[b]	5.0 mg. twice daily
124	3.9	28	Human pituitary gonadotropin concentrate[b]	0.5 mg. twice daily
91	4.1	45	Human pituitary gonadotropin concentrate[b]	0.05 mg. twice daily
172	4.1	3	Equine LH[c]	5.0 mg. twice daily
165	5.0	7	Ovine LH[d]	2.0 mg. twice daily
167	5.7	8	Ovine LH[e]	0.5 mg. twice daily
182[g]	3.9	4	Ovine LH[d]	5.0 mg. twice daily
	4.1	6	Equine LH[c]	5.0 mg. twice daily
151[g]	3.1	16	Ovine LH[e]	4.0 mg. once daily
	3.2	18	HCG[f]	250 I.U. twice daily
89	4.0	40	HCG[f]	500 I.U. daily (7 days)
				500 I.U. twice daily (7 days)

[a] All treatment given for 14 days. Doses of pituitary LH are given as mg. equivalent of Armour Standard No. 227-80 with the exception of the human material.
[b] Merck No. 581, 037-0-3.
[c] Armour Veterinary horse LH—No. R377279.
[d] NIH—LH—S—1.
[e] Armour Standard No. 227-80.
[f] Human Chorionic Gonadotropin—Parke, Davis No. 559548.
[g] Two experiments performed in this animal at an interval of 2 months.

RESULTS AND DISCUSSION

The appearance of the testis and seminal vesicle of a normal, sexually mature rhesus monkey is presented in Figure 1 for purposes of comparison with those removed from hypophysectomized animals prior to and following gonadotropin treatment. The interstitial cells of Leydig are clearly evident and contain small amounts of sudanophilic material (Figs. 1A and B). Intratubular sudanophilia is pronounced (Fig. 1B). The seminal vesicle (Fig. 1C) is composed of highly branched tubular elements lined with tall columnar epithelial cells possessing basal nuclei and relatively large quantities of cytoplasm.

Human chorionic gonadoptropin (HCG). Our initial experiments were designed to determine, in a qualitative sense, the conditions under which

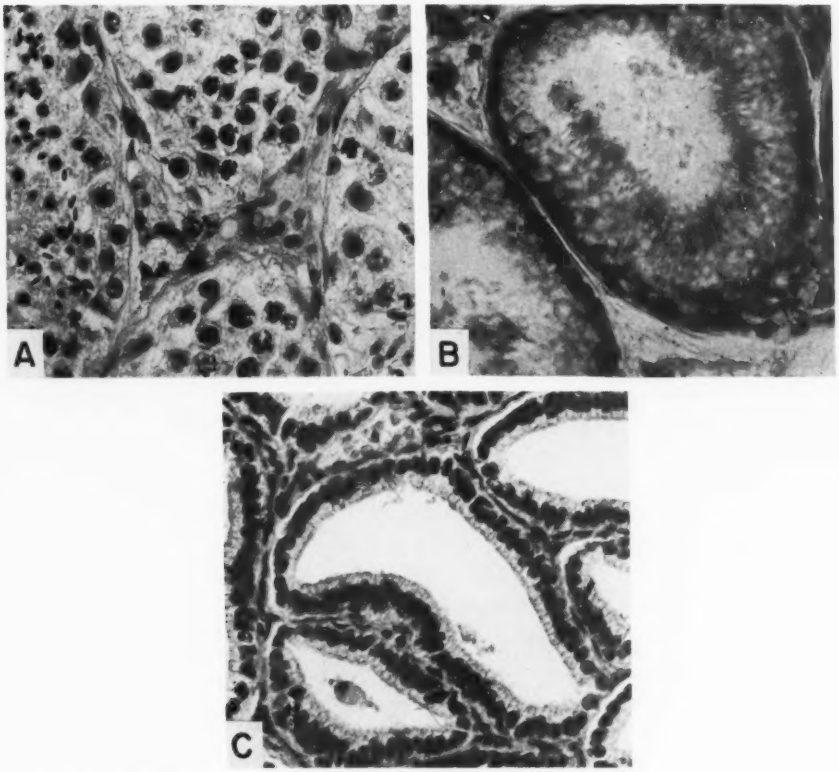

FIG. 1. Normal, mature male rhesus monkey
A—Testis, H & E×500
B—Testis, Sudan black B×270
C—Seminal vesicle×500
The above magnifications also apply to all subsequent Figures.

the effects of interstitial cell stimulating preparations could be unequivocally detected in the hypophysectomized male monkey. To this end two hypophysectomized animals were treated with HCG (Monkeys 89 and 151; see Table 1) The results illustrated in Figure 2 prompted us to adopt the treatment regimen outlined in the Methods section. Eighteen months after hypophysectomy, the control biopsies of the testis (Fig. 2A) of an animal (Monkey 151) with a chronological age of approximately 4 years reveal the histological appearance previously described in sexually immature animals (8). The interstitial cells are difficult to identify with certainty and spermatogenesis is absent. The tubular diameter is considerably smaller than that in normal controls. Sudanophilic droplets are absent in the tubules and the interstitial cells (Fig. 2C). The epithelium of the seminal vesicle of the same animal subjected to biopsy at the same time presents the typical castrate appearance characterized principally by the paucity of cytoplasm. (Fig. 2B)

Treatment of this animal with HCG for 14 days resulted in the appearance of distinguishable interstitial cells in the testis (Fig. 2D) and of sudanophilic droplets in the intertubular spaces (Fig. 2F). No discrete sudanophilia was evident within the tubules. While the localization of these droplets in the treated hypophysectomized monkeys is difficult to ascertain, they appear to represent sudanophilic material within the interstitial cells of Leydig. This conclusion is supported by the finding that in the fetal testis (128th day of gestation) where the interstitial cells are numerous and clearly delineated, intracellular sudanophilic droplets are readily demonstrable (Fig. 3). It is of interest in this connection that the fetal testis closely resembles that of the hypophysectomized monkey treated with HCG in that the fetal monkey has minimal endogenous gonadotropin secretion, yet is subjected to the influence of placental and/or maternal pituitary hormones which stimulate the interstitial cells, but not the spermatogenic elements of the tubules.

The glandular epithelium of the seminal vesicle in the HCG treated hypophysectomized animals was clearly stimulated (Fig. 2E) as evidenced by an accretion in cell height and the appearance of the cytoplasmic characteristics associated with the action of androgens (7). These effects were grossly exaggerated in the animal (Monkey 89) treated with the higher doses of HCG.

These histological evidences of gonadotropin and consequent androgenic activity were accompanied by a striking scrotal edema which became evident during the first week of treatment, a partial descent of the testes and pigmentation of the perineum. In these and subsequent experiments frequent erections were noted during hormone treatment, a phenomenon never observed prior to therapy.

Human Pituitary Gonadotropin. The effects of a relatively crude human

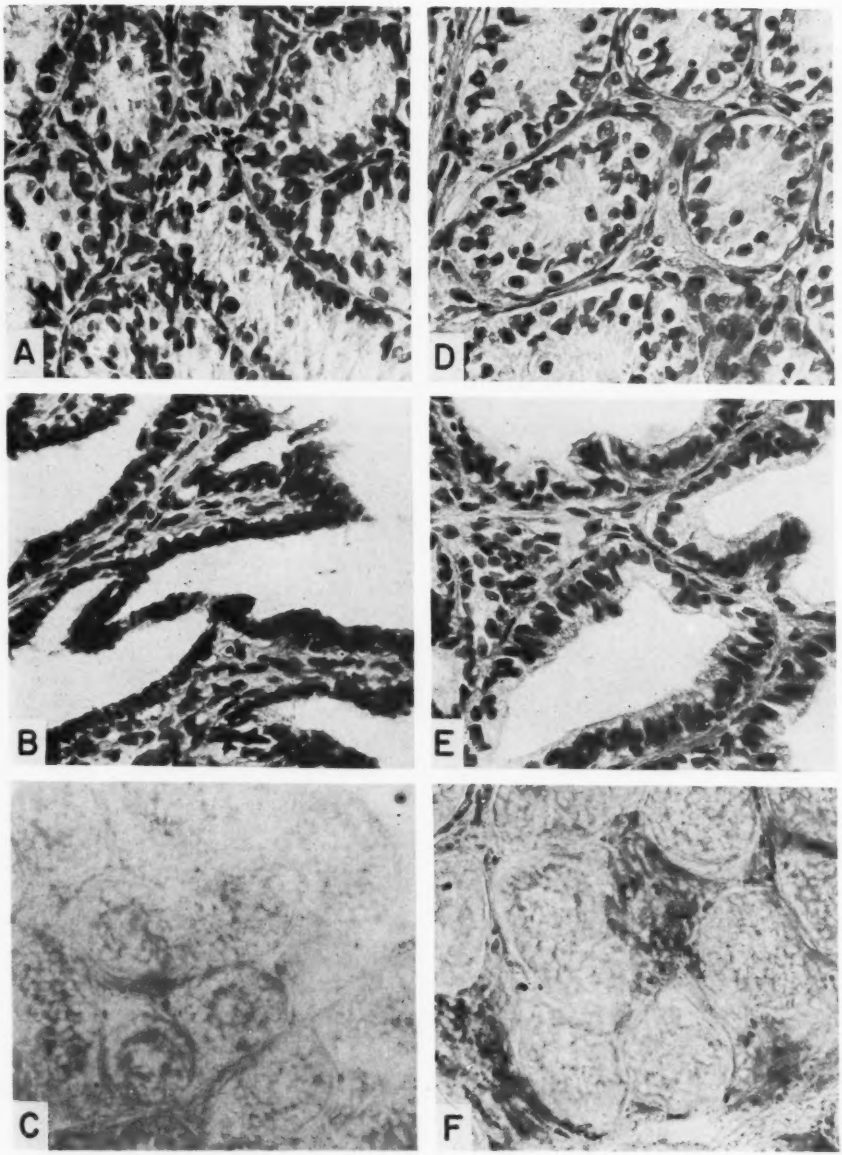

FIG. 2. Testicular and seminal vesicle biopsies from a hypophysectomized monkey (No. 151) before (A–C) and after (D–F) treatment with HCG (250 I.U. ×2/day for 14 days). A and D: Testis H & E. B and E: Seminal vesicle, H & E. C and F: Testis, Sudan black B. Compare with Fig. 1.

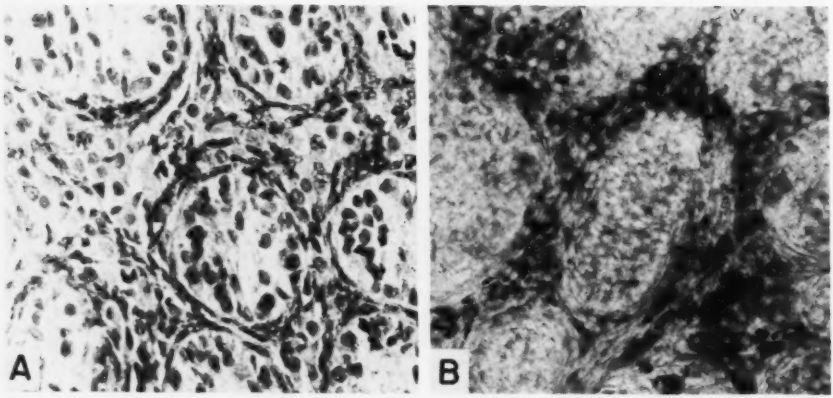

FIG. 3. Fetal Testis (128th day of gestation) A. H & E.; B. Sudan black B. Note localization of sudanophilic droplets in interstitial cells and general resemblance to hypophysectomized animals treated with HCG or LH.

pituitary gonadotropin concentrate in three hypophysectomized animals are illustrated in Figures 4, 5 and 6. The lowest dose of this preparation (0.1 mg. daily in two divided doses for 14 days; Fig. 6) effected testicular stimulation in terms of the criteria utilized in this study. The action of the human pituitary material on the interstitial cells did not differ qualitatively from that of the human placental hormone.

Ovine Pituitary Luteinizing Hormone. Two ovine LH preparations were studied: the Armour Standard No. 227–80 and the material distributed by the Endocrinology Study Section of the National Institutes of Health (NIH-LH-S-1). In one animal (Monkey 151) the Armour preparation was inactive when administered as a single daily dose of 4 mg. for 14 days. When given in two divided daily doses of 0.5 mg. each for the same length of time, however, testicular stimulation was observed (Fig. 7). Unequivocal responses to the NIH material were also observed at doses of 2 and 5 mg. given twice daily (Fig. 8).

Equine pituitary luteninizing hormone. This relatively crude preparation administered in two daily doses of 5 mg. each for 14 days effected an increase in androgen production as evidenced by the hypertrophy of the seminal vesicle epithelium although the intertubular sudanophilia in the stimulated testis did not differ, in a significant fashion, from control (Fig. 9). In one animal (Monkey 182) in which the equine preparation was given 2 months following a course of ovine LH administration, no response to the equine hormone was observed. It may be that this failure to respond to a dose of equine LH found effective in another monkey is attributable to the formation of antibodies against the ovine preparation which cross

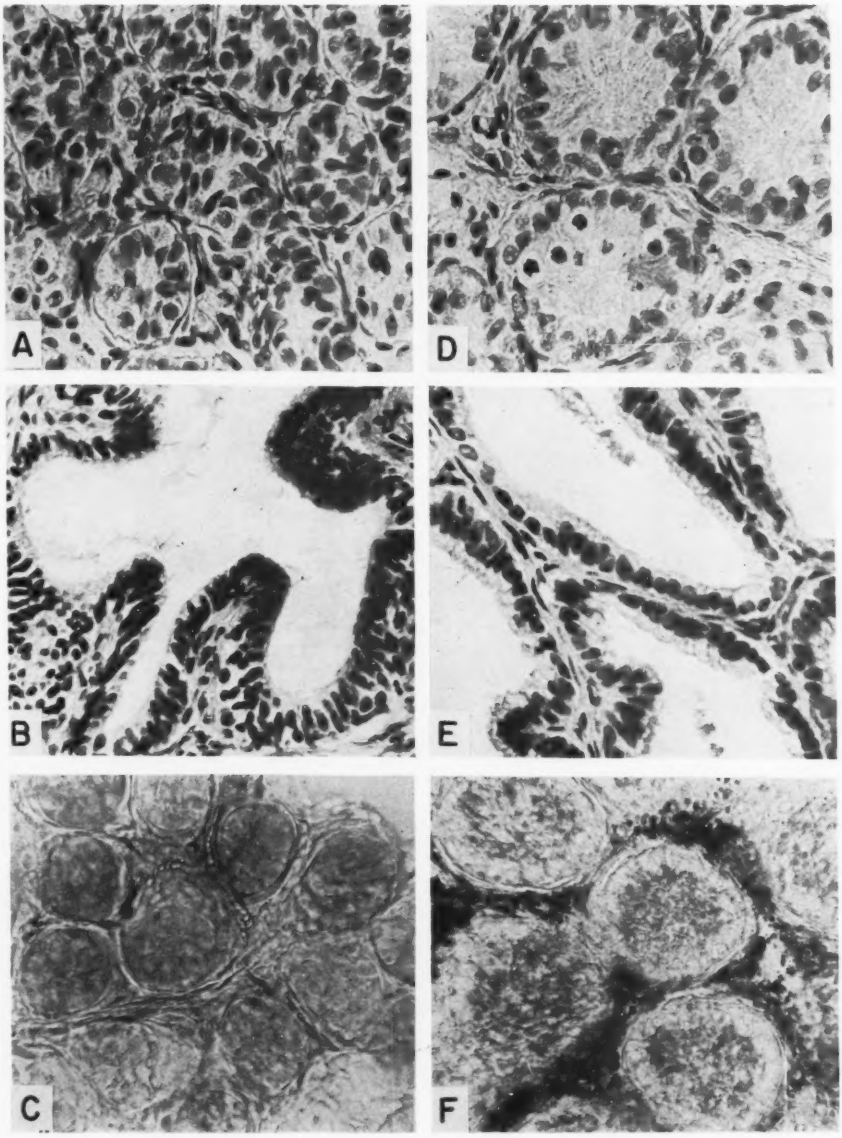

FIG. 4. Testicular and seminal vesicle biopsies from a hypophysectomized monkey (No. 166) before (A–C) and after (D–F) treatment with a human pituitary gonadotropin preparation (5 mg. ×2/day for 14 days). See Fig. 2 for legend.

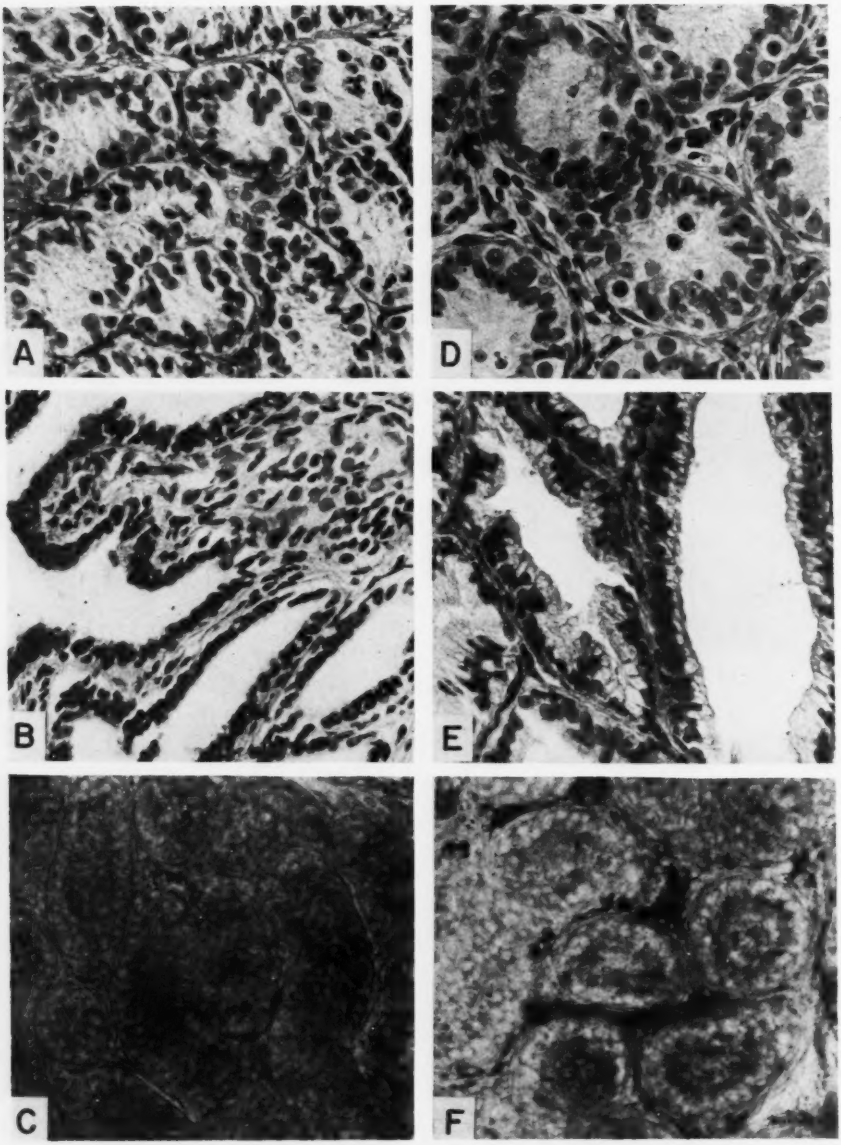

FIG. 5. Testicular and seminal vesicle biopsies from a hypophysectomized monkey (No. 124) before (A–C) and after (D–F) treatment with a human pituitary gonadotropin preparation (0.5 mg. $\times 2$/day for 14 days). See Fig. 2 for legend.

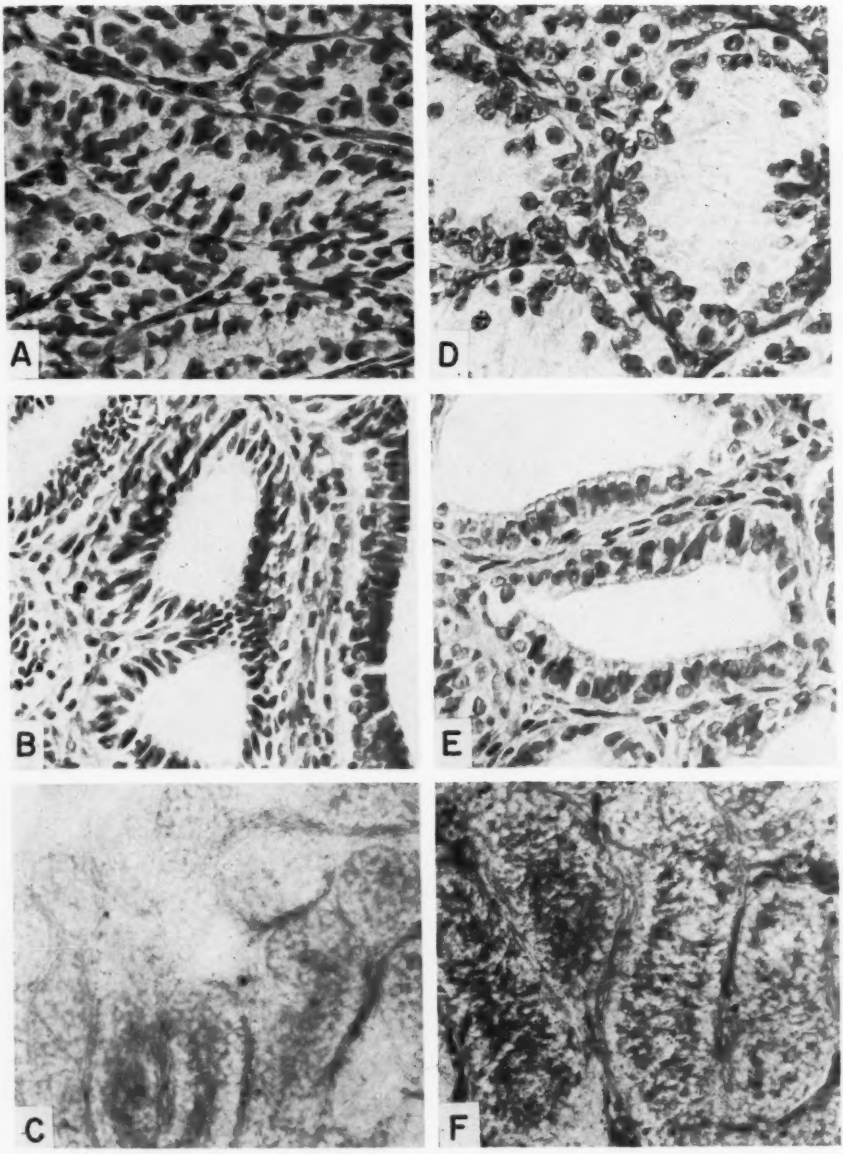

FIG. 6. Monkey No. 91. Testicular and seminal vesicle biopsies before (A–C) and after (D–F) treatment with a human pituitary gonadotropin preparation (0.05 mg. ×2/day for 14 days). See Fig. 2 for legend.

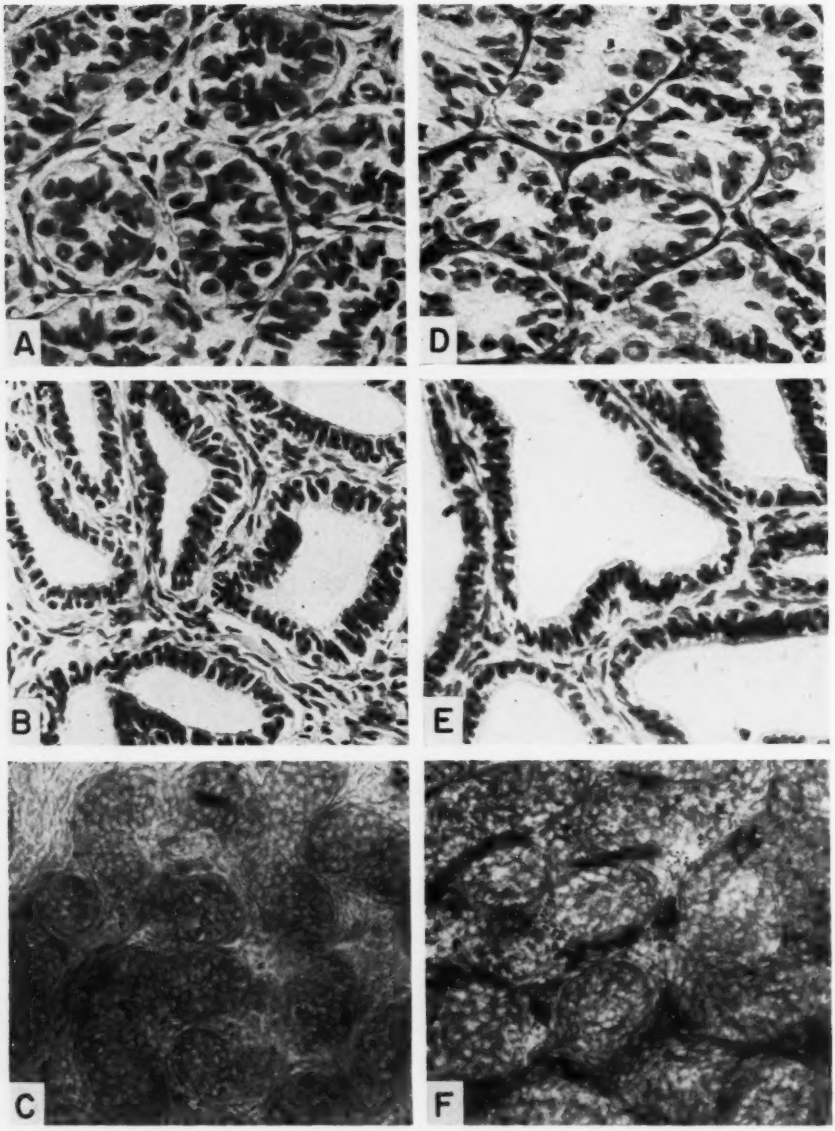

FIG. 7. Response of the testis and seminal vesicle of a hypophysectomized monkey (No. 167) to treatment with ovine LH (0.5 mg.×2/day for 14 days). See Fig. 2 for legend.

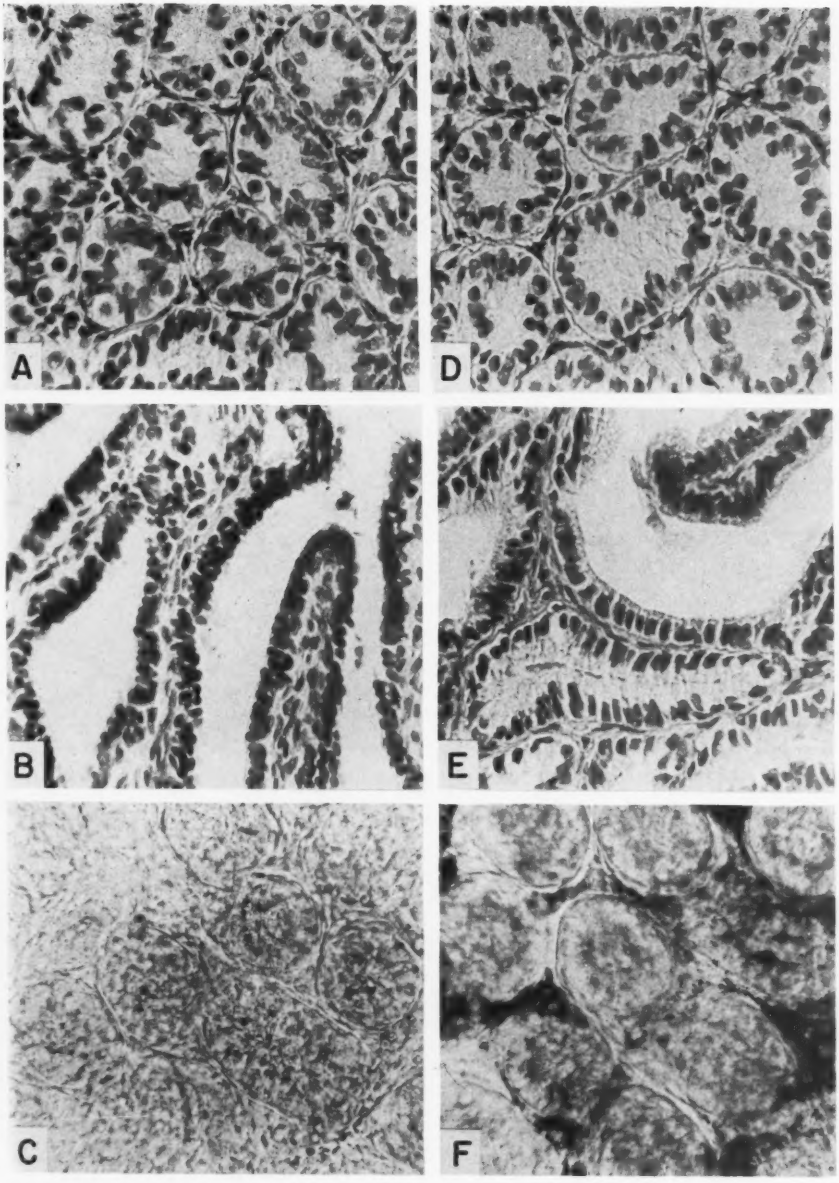

FIG. 8. Monkey No. 182. Testicular and seminal vesicle biopsies before and after treatment with ovine LH (5 mg.×2/day for 14 days). See Fig. 2 for legend.

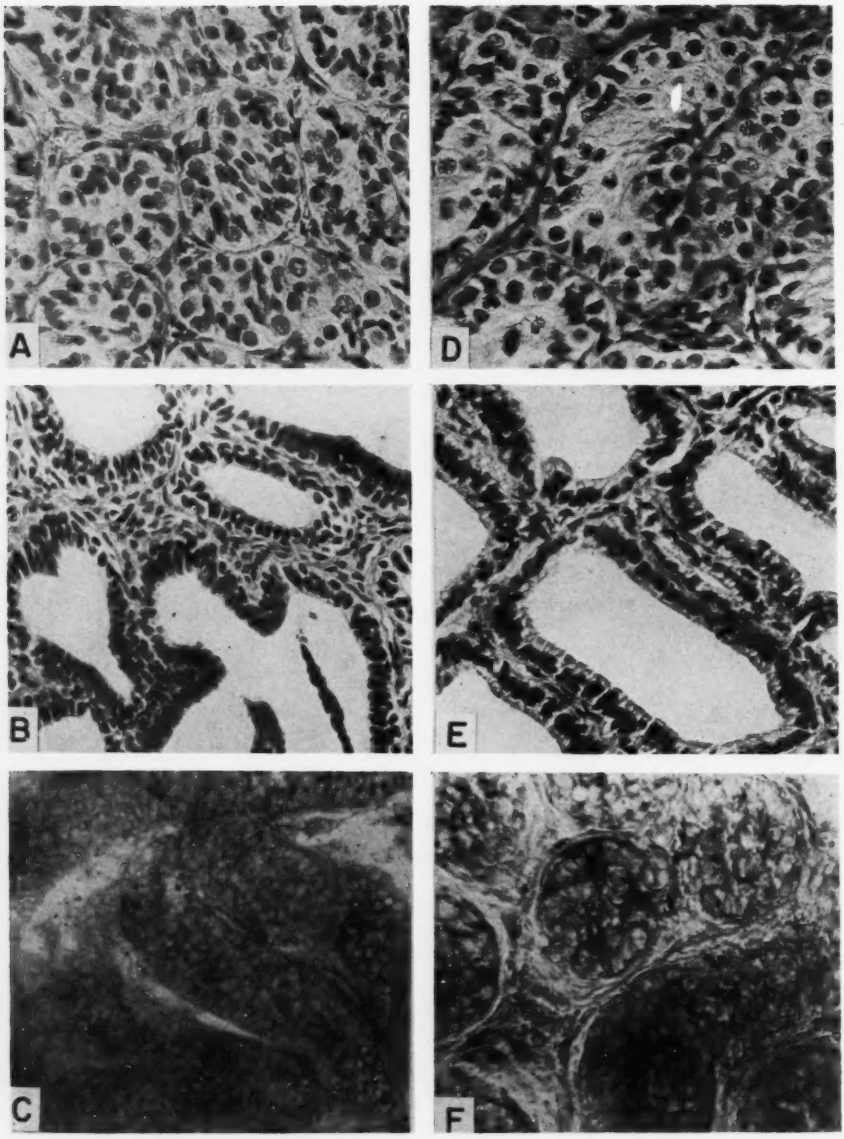

FIG. 9. Response of the testis and seminal vesicle of a hypophysectomized monkey (No. 172) to an equine LH preparation (5 mg.×2/day for 14 days). See Fig. 2 for legend.

reacted with the equine hormone or to the fact that the dose utilized was near the minimal effective dose.

The foregoing experiments lead to the conclusion that lutenizing hormone preparations of sub-primate origin can be physiologically effective in the hypophysectomized rhesus monkey. While the data suggest quantitative differences in the efficacy of primate and sub-primate preparations in the monkey, no definitive conclusions can be reached in this regard because of the small number of observations involved, but the fact remains that an absolute "species specificity" similar to that described for the action of growth hormone cannot be attributed to the luteinizing (interstitial cell stimulating) hormone.

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THE METABOLISM OF ADRENOCORTICAL STEROIDS BY HUMAN TISSUES[1]

ROBERT A. MEIGS[2] AND LEWIS L. ENGEL[3]

John Collins Warren Laboratories of the Huntington Memorial Hospital of Harvard University at the Massachusetts General Hospital, and the Department of Biological Chemistry, Harvard Medical School, Boston, Massachusetts

ABSTRACT

Adrenocortical steroids were incubated with preparations of human liver and term placenta and the metabolic products were isolated and characterized. Human liver homogenates interconverted cortisol and cortisone and reduced the latter substance to 5β-dihydrocortisone, tetrahydrocortisone and 20-dihydrocortisone. Tetrahydrocortisone was oxidized to 5β-dihydrocortisone and reduced to cortolone. Δ^4-Reductase (5β) activity of human liver was found only in the soluble fraction while 3α-ol dehydrogenase activity against tetrahydrocortisone and 11β-ol dehydrogenase activity against cortisol were found concentrated in microsomal fractions. Placenta slices oxidized cortisol to cortisone, tetrahydrocortisone to 5β-dihydrocortisone, reduced tetrahydrocortisone to cortolone and, as in the case of liver, 3α-ol and 11β-ol dehydrogenases were associated with the microsomes. Further study of the microsomal dehydrogenase activities showed them to be reversible and to utilize pyridine nucleotides as cofactors. Di- and triphosphopyridine nucleotides were equally effective in stimulating these activities of placental microsomes while with liver microsomes their relative effectiveness varied with the substrate studied. In addition to cortisol and tetrahydrocortisone, a variety of other 3α, 3β-, and 11β-hydroxysteroids were oxidized to the corresponding ketones by microsomes from both liver and placenta. These findings on the metabolic properties and biochemical characteristics of human tissues have been compared with the results obtained in studies of other species.

THE main pathways of metabolism of the adrenocortical steroids in the intact human involve one or more of the following chemical reactions: 1) saturation of the 4-ene structure, 2) reversible reduction of the 3-ketone,

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[2] Predoctoral Fellow of the National Science Foundation. This work is taken from a thesis submitted in November 1958 to the Division of Medical Sciences, Graduate School of Arts and Sciences, Harvard University in partial fulfillment of the requirements for the degree of Doctor of Philospohy. A preliminary report has appeared in *Fed. Proc.* **17**: 274. 1958.

[3] Permanent Faculty Fellow of the American Cancer Society.

3) interconversion of 11β-hydroxyl and 11-ketone functions, 4) reduction of the 20-ketone, 5) oxidative removal of the side chain, and 6) conjugation of hydroxyl functions with glucuronic acid (1). Although all of these conversions have been demonstrated and examined in *in vitro* experiments, tissues from lower animals have almost invariably been used (1). Because of possible species differences, information on the properties of human tissues was considered necessary before any correlation of *in vivo* and *in vitro* findings could be attempted. In this study, human liver and placenta were examined for their capacity to metabolize the corticosteroids. The biochemical characteristics of these tissues were compared with previous findings using tissues from other mammalian species.

MATERIALS AND METHODS

Steroids. Steroids used as substrates were generously provided by the Merck, Upjohn, Ciba, Syntex, and Schering Companies and were chromatographically pure.

Solvents. Analytical quality or redistilled solvents were used throughout for extractions and chromatography.

Cofactors. DPN[4] and TPN were commercial preparations. DPNH and TPNH were prepared by chemical reduction of the oxidized nucleotides by the method of Kaplan *et al.* (2). Alternatively TPNH was generated during incubations by the addition of 0.2 μmole TPN, 9 μmoles glucose-6-phosphate and 0.75 mg. glucose-6-phosphate dehydrogenase (Sigma Chemical Co., Practical type II) per milliliter of incubation mixture.

Tissues. Human liver was fresh, normal material obtained at partial hepatectomy through the courtesy of Drs. G. A. Donaldson and G. S. Richardson of the Surgical Service, Massachusetts General Hospital. Human term placenta was obtained through the courtesy of Dr. C. A. Villee and his staff at the Boston Lying-In Hospital.

Tissue Preparations and Incubations. Tissues were rapidly cooled in ice and all subsequent operations, prior to incubation, were carried out at 0–4° C. Placenta slices were cut on a Stadie-Riggs microtome, suspended in Krebs' phosphate saline, pH 7.4 (3), blotted and 250–500 mg. suspended in 2–3 ml. of fresh medium and incubated with 300–500 μg of steroid substrate for 3 hours. Minced liver was homogenized in 3 volumes of 0.05 M sodium phosphate, pH 7.4, 0.04 M nicotinamide (homogenate I) or 0.05 M glycine, pH 9.0, 0.04 M nicotinamide (homogenate II) and the resulting suspensions cleared of debris and larger particles by centrifugation at 9,000 g. for 10 minutes. Homogenate I in 2 ml. portions supplemented with 1.7 mg. of TPNH was incubated with 250 μg of steroid sub-

[4] DPN, TPN, DPNH and TPNH are oxidized and reduced di- and triphosphopyridine nucleotides respectively. EDTA is ethylenediamine tetraacetic acid. The following trivial names for steroids are employed: cortisol, 11β,17,21-trihydroxy-4-pregnene-3,20-dione; cortisone, 17,21-dihydroxy-4-pregnene-3,11,20-trione; 5β-dihydrocortisone; 17,21-dihydroxy-5β-pregnane-3,11,20-trione; 20-dihydrocortisone, 17,20ξ,21-trihydroxy-4-pregnene-3,11-dione; tetrahydrocortisone, 3α,17,21-trihydroxy-5β-pregnane-11,20-dione; tetrahydrocortisol, 3α,11β,17,21-tetrahydroxy-5β-pregnane-20-one; cortolone, 3α,17,20ξ,21-tetrahydroxy-5β-pregnane-11-one; α-cortolone, 3α,17,20α,21-tetrahydroxy-5β-pregnane-11-one; β-cortolone, 3α,17,20β,21-tetrahydroxy-5β-pregnane-11-one; androsterone, 3α-hydroxy-5α-androstane-17-one; etiocholanolone, 3α-hydroxy-5β-androstane-17-one; dehydroepiandrosterone, 3β-hydroxy-5-androstene-17-one; 17-hydroxypregnenolone, 3β,17-dihydroxy-5-pregnene-20-one.

strate for 90 minutes. Similar volumes of homogenate II supplemented with 2 mg. of DPN were incubated with 250 μg of steroid substrate for 90 minutes. All incubations were carried out at 37° C in an air atmosphere with shaking. Steroid substrates were added as propylene glycol solutions containing 5 mg./ml.

Extraction of Incubation Mixtures

Tissue Slices: Incubation mixtures were diluted with 10 ml. of acetone, filtered and the denatured slices ground with sand in a mortar. The pulverized tissue was repeatedly extracted with hot acetone and the extracts washed through the filter until approximately 50 ml. were collected. The extract was evaporated to dryness *in vacuo*. The residue was suspended in 6 ml. of water and extracted with four 6 ml. portions of ethyl acetate. The combined ethyl acetate extracts were washed once with 2 ml. of water and evaporated. The residue was dissolved in 5 ml. of aqueous 90% methanol and extracted with four 5 ml. portions of hexane. The remaining methanol fraction contained the partially purified steroid extract.

Homogenate and Cell Fraction Incubations: Incubation mixtures were extracted twice with ten volumes of methylene chloride. The combined extracts were washed once with one-tenth volume of water and evaporated. The residue was partitioned between 5 ml. of aqueous 90% methanol and 15 ml. of hexane. The methanol layer contained the partially purified steroid extract.

Fractionation of Partially Purified Steroid Extracts. Crude steroid extracts were fractionated by chromatography on Whatman No. 2 paper at 37° C using the techniques of Bush (4). The following systems were used: System I: Toluene 9, ethyl acetate 1, methanol 6, water 4; System II: Toluene 3.3, ligroin 6.7, methanol 7, water 3.

Characterization of Steroids. Steroid incubation products were characterized by their chromatographic mobilities and chemical reactivities on paper and compared with standard compounds chromatographed on the same paper. Compounds containing the Δ^4-3-keto structure were detected by their absorption of ultraviolet light. Steroids having an α-ketol side chain were detected by their reduction of Blue Tetrazolium. The Zimmermann reagent (alkaline m-dinitrobenzene) (5) was used to detect ketosteroids. Periodic acid oxidation followed by the Zimmerman reaction was employed for the detection of 17,20-glycols (6). Chromatographically purified C_{21} steroids were further characterized by elution and oxidation to C_{19} steroids with either sodium bismuthate (7) or periodic acid (8). The resulting oxidation products were also characterized by their chromatographic mobilities and chemical reactivities toward the spray reagents.

Quantitative Methods. All enzymatic activity was assayed by measurements of the steroid product produced. Products containing the α-ketol side chain were chromatographed on paper, sprayed with Blue Tetrazolium and the resulting colored diformazan determined by a modification of the method of Cope and Hurlock (9) in which ethyl acetate was substituted for hydrochloric acid in the eluting solvent. 17-Ketosteroids were determined by elution from chromatograms and application of the Wilson micro-Zimmermann procedure (10). Protein was assayed by the turbidometric method of Kunitz (11).

Nucleotide Pyrophosphatase Treatment of Microsomes. Fourteen milligrams of washed microsomal protein suspended in 12 ml. of 0.05 M sodium phosphate, pH 7.1 were incubated for 10 minutes at 37° C with 1.5 mg. of dried rattlesnake venom (Crotalus adamanteus, Ross Allen's Reptile Institute, Silver Springs, Florida). The suspension was cooled and the microsomes were sedimented at 105,000 g. for 1 hour and stored at −10° C. Prior to incubation, pyrophosphatase-treated microsomes were washed in 0.05 M glycine, pH 8.8, 0.001 M EDTA.

EXPERIMENTAL AND RESULTS

Metabolic Activities of Human Tissues and Characterization of Reaction Products

The metabolic transformations of cortisone, cortisol, and tetrahydrocortisone observed with preparations of human liver and placenta are listed in Table 1. The characterization of the products is described below.

5β-Dihydrocortisone was characterized by its mobility in system I, its reduction of Blue Tetrazolium, its lack of absorption of ultraviolet light, its distinctive reddish reaction with the Zimmermann reagent and by its oxidation by bismuthate to a product corresponding to 5β-androstane-3,11,17-trione in mobility in system II and in its reactivity toward the

TABLE 1. METABOLISM OF ADRENOCORTICAL STEROIDS BY HUMAN TISSUES

Reaction	Tissue	Preparation*
Reduction of 4-ene group		
Cortisone→5β-dihydrocortisone	Liver	Homogenate I
Reduction of 20-ketone		
Cortisone→20-dihydrocortisone	Liver	Homogenate I
Tetrahydrocortisone→cortolone	Liver	Homogenate I
Tetrahydrocortisone→cortolone	Placenta	Slices
Oxidation-reduction at position 3		
Cortisone→tetrahydrocortisone	Liver	Homogenate I
Tetrahydrocortisone→5β-dihydrocortisone	Liver	Homogenate II
Tetrahydrocortisone→5β-dihydrocortisone	Placenta	Slices
Oxidation-reduction at position 11		
Cortisone→cortisol	Liver	Homogenate I
Cortisol→cortisone	Liver	Homogenate II
Cortisol→cortisone	Placenta	Slices

* Described in detail in Methods section of text.

Zimmermann reagent. Acetylation of the product formed during incubations yielded a product which had the same migration rate as 21-acetoxy-5β-dihydrocortisone in system II and which was separable from the 5α epimer.

20-Dihydrocortisone had the same mobility as 17,20β,21-trihydroxy-4-pregnene-3,11-dione (Reichstein's Substance U), absorbed ultraviolet light, failed to reduce Blue Tetrazolium and gave a positive test for the 17,20-glycol structure. Its periodic acid oxidation product corresponded to 4-androstene-3,11,17-trione in mobility in system II. It also absorbed ultraviolet light and reacted with the Zimmermann reagent. Although the incubation product corresponded to the 20β-dihydrocortisone in its chromatographic mobility, it is unlikely that the system used is capable of resolving the 20α and β epimers. Since the 20α compound was unavailable for comparison, the configuration of the product at C-20 could not be established.

Cortolone had the same mobility as β-cortolone in system I. It failed to reduce Blue Tetrazolium but gave a positive test for the 17,20-glycol structure. The periodic acid oxidation product corresponded to 3α-hydroxy-5β-androstane-11,17-dione both in its mobility in system II and in its reactivity toward the Zimmermann reagent. As with the previous 20-hydroxy compound, the configuration at C-20 could not be established since 20α and β epimers were unlikely to have been resolved and no α-cortolone was available for comparison.

Tetrahydrocortisone had the correct mobility in system I and was distinguishable from the 3α,5α tetrahydro derivatives of cortisone. It did not absorb ultraviolet light but reduced Blue Tetrazolium. It was converted by bismuthate oxidation to a product corresponding in its mobility in system II and in its reactivity with the Zimmermann reagent to 3α-hydroxy-5β-androstane-11,17-dione.

Cortisol formed in incubations absorbed ultraviolet light, reduced Blue Tetrazolium and had the same mobility in system I as the standard compound. It was converted by sodium bismuthate oxidation to a product corresponding to 11β-hydroxy-4-androstene-3,17-dione in chromatographic mobility in system II. This material also absorbed ultraviolet light and gave a positive Zimmermann reaction.

Cortisone absorbed ultraviolet light, reduced Blue Tetrazolium and migrated at the same rate as the standard in system I. On bismuthate oxidation it was converted to a product corresponding to 4-androstene-3,11,17-trione in mobility in system II and in reactivity with the Zimmermann reagent.

Localization of Enzymatic Activities in Cell Fractions

Liver homogenate I (see Methods section) was separated into microsomal and soluble fractions. (Mitochondria had been removed in the initial preparation of the homogenate.) Microsomes were sedimented at 105,000 g. for 1 hour, washed once and resuspended in fresh medium to the volume of the original homogenate. The soluble fraction was the supernatant solution remaining after centrifugation of homogenate I at 105,000 g. for 2 hours. Cell fractions were incubated with cortisone at the same time under the conditions previously described for the whole homogenate. The ring-A reduction to 5β-dihydrocortisone and tetrahydrocortisone observed with the homogenate preparation also occured in the soluble fraction, but not in the microsomal fraction.

Liver homogenate II was separated into microsomal and soluble fractions in the same manner as was homogenate I. Cell fractions were incubated with cortisol and with tetrahydrocortisone, at the same time and under the same conditons used for the intact homogenate. The distribution of 11β-ol dehydrogenase activity and 3α-ol dehydrogenase activity among the preparations is shown in Table 2.

TABLE 2. HYDROXYSTEROID DEHYDROGENASE ACTIVITIES IN FRACTIONATED LIVER HOMOGENATE II

Dehydrogenase	Substrate	Ketosteroid formed ($\times 10^{-2}$ μmole)		
		Homogenate II	Microsomes	Soluble fraction
11β-ol	Cortisol	7.0	18.0	1.1
3α-ol	Tetrahydrocortisone	5.5	15.8	0.83

Cell fractions were maintained at a volume equivalent to that of the original homogenate. Incubation conditions are described in the text.

Homogenates of placenta were made in 2 volumes of 0.25 M sucrose, 0.04 M nicotinamide. The total particulate material was sedimented at 105,000 g. for 1 hour, washed and resuspended to the original homogenate volume. A soluble fraction was prepared by centrifuging the homogenate for 2 hours at 105,000 g. and retaining the clear supernatant solution. Two milliliter aliquots of intact homogenate or homogenate fractions were buffered to pH 8.9 by the addition of 7.5 mg. of glycine and 1N NaOH and incubated with 2 mg. of DPN and 250 μg of cortisol or tetrahydrocortisone for 2 hours. The distribution of 3α-ol and 11β-ol dehydrogenase activities in the placental fractions is given in Table 3. Placental particulate matter, fractionated according to the procedures of Schneider and Hogeboom (12), was found to be predominantly microsomal. Mitochondria, sedimented at 9,000 g. and staining with Janus Green, were present in only small amounts and had from 1/10–1/30 the dehydrogenase activity of the microsomal fraction.

Characteristics of Microsomal Hydroxysteroid Dehydrogenases

Cofactor Requirements. The hydroxysteroid dehydrogenase activites of washed liver microsomes were determined with and without the addition of pyridine nucleotides. The results are given in Table 4. Stimulation of endogenous microsomal activity by one or both of the nucleotides was always observed. In an attempt to reduce activity due to endogenous cofactors not readily removed by washing or dialysis, microsomes were pretreated (see Methods) with snake venom containing a pyrophosphatase capable of cleaving bound nucleotides (13). The results of incuabtions utilizing

TABLE 3. HYDROXYSTEROID DEHYDROGENASE ACTIVITIES IN FRACTIONATED PLACENTAL HOMOGENATES

Dehydrogenase	Substrate	Ketosteroid formed ($\times 10^{-2}$ μmole)		
		Whole homogenate	Particles	Soluble fraction
11β-ol	Cortisol	9.7	7.2	1.7
3α-ol	Tetrahydrocortisone	15.0	10.0	4.4

Cell fractions were maintained at a volume equivalent to that of the original homogenate. Incubation conditions are described in the text.

TABLE 4. COFACTOR STIMULATION OF HYDROXYSTEROID DEHYDROGENASE ACTIVITIES OF HUMAN LIVER MICROSOMES

Dehydrogenase	Substrate	Ketosteroid formed (μg)		
		No cofactor	DPN	TPN
3α-ol	Tetrahydrocortisone	10	34	10
	Tetrahydrocortisone*	0	25	0
	Androsterone	7.5	17.5	15
	Androsterone*	0	25	13
	Etiocholanolone*	0	10	5
11β-ol	Cortisol	37	55	55
	Cortisol*	18	30	50

* Incubations using microsomes pretreated with snake venom nucleotide pyrophosphatase. All incubations were carried out at 37° C in 2 ml. 0.05 M glycine, pH 8.8, 0.001 M EDTA. 1.5 Mg. of DPN or TPN was added per flask as indicated. Microsome protein content varied from 1.4–4.0 mg. and incubation time from 10–20 minutes in the separate experiments but were constant for incubations with a given substrate.

venom-treated microsomes are also given in Table 4. Endogenous 11β-ol dehydrogenase activity was greatly reduced in these preparations. Endogenous activity against 3α-hydroxysteroids was completely eliminated, thus permitting the demonstration of an absolute requirement for the nucleotide cofactors.

Hydroxysteroid dehydrogenase activity was not observed with washed placental microsomes in the absence of added pyridine nucleotides. In incubations similar to those with liver microsomes but carried out for longer time periods, DPN and TPN were equally effective in promoting the dehydrogenation of cortisol to cortisone and of tetrahydrocortisone to dihydrocortisone by placental microsome preparations.

Reversibility. The hydroxysteroid dehydrogenase activities of hepatic and placental microsomes were also examined for reversibility. Two to four milligrams of microsome protein were suspended in 2 ml. of 0.05 M sodium phosphate, pH 7.1 and incubated with 250 μg of steroid substrate and 1.5 mg. of DPNH.

Liver microsomes converted 5β-dihydrocortisone to 3α,5β-tetrahydrocortisone. 5α-Dihydrocortisone was reduced to a mixture of 3α,5α-tetrahydrocortisone and 3β,5α-tetrahydrocortisone. 5β-Androstane-3,17-dione and 5α-androstane-3,17-dione were both converted to mixtures of 3α- and 3β-hydroxysteroids. Cortisone was reduced to cortisol in incubations in which the TPNH generating system was substituted for DPNH.

Placental microsomes also reduced 5β-dihydrocortisone to 3α,5β-tetrahydrocortisone. 5α-Dihydrocortisone, 5α-androstane-3,17-dione and 5β-androstane-3,17-dione were all reduced only to 3β-hydroxy products.

Substrate Requirements. Microsomes from both liver and placenta showed 3α-ol dehydrogenase activity against tetrahydrocortisone, tetrahydrocortisol, β-cortolone, 3α,11β-dihydroxy-5β-androstane-17-one, etiocholano-

lone and androsterone. 11β-ol Dehydrogenase activity against cortisol, tetrahydrocortisol, 11β-hydroxy-4-androstene-3,17-dione, and 3α,11β-dihydroxy-5β-androstane-17-one was also demonstrated. Liver microsomes also oxidized the 3β-hydroxyl groups of 3β-hydroxy-5β-androstane-17-one, 3β-hydroxy-5α-androstane-17-one, and 3β,17,21-trihydroxy-5α-pregnane-11,20-dione. Placental microsomes possessed 3β-ol dehydrogenase activity against 3β-hydroxy-5α-androstane-17-one, dehydroepiandrosterone, and 17-hydroxypregnenolone.

DISCUSSION

By measuring a decrease of ultraviolet light absorption at 240 mμ, Reaven (14) demonstrated that human liver slices could modify the ring A of cortisol, corticosterone, deoxycorticosterone and aldosterone. Romani *et al.* (15) detected several polar metabolites which no longer absorbed ultraviolet light after incubation of aldosterone with human liver homogenates but these products were not defined chemically. The ring A reduction products of cortisone obtained in the present study of human liver, 5β-dihydrocortisone and tetrahydrocortisone, both have the 5β configuration and are normal metabolites of cortisone in the intact human.

In contrast, incubations of corticosteroids with unfractionated rat liver preparations have yielded only 5α reduction products (see references in 1). Studies of the *in vitro* metabolism of progesterone have also revealed this difference in ring A metabolism by livers of the two species. Human liver suspensions produce progesterone metabolites having predominantly the 5β configuration (16), while rat liver incubations yield predominantly 5α products (17). The Δ^4-reductase (5β) activity of human liver does resemble that of rat liver in being located in the soluble fraction of homogenates (18, 19).

Sex differences in both the steric course and rates of ring A reduction of the corticosteroids by rat liver preparations have been reported (20, 21). Limitations in tissue supply prevented any conclusions as to the influence of this factor in human liver.

The 3α-ol dehydrogenase activity against tetrahydrocortisone in human liver was found to be associated with microsomes and to utilize only DPN as a cofactor. Rat liver microsomes are capable of reducing the 3-ketone of corticosteroids to 3α-hydroxy products but the cofactor requirements for this reaction have not been established (18, 22). In addition, rat liver contains a soluble, relatively unspecific 3α-ol dehydrogenase which utilizes either DPN or TPN and which is active against C_{19} as well as C_{21} 3α-hydroxysteroids (23). Human liver microsomes also display 3α-ol dehydrogenase activity against a variety of steroid substrates. However, the observation that TPN can replace DPN only in the oxidation of the C_{19} 3α-hydroxysteroids suggests that more than one enzyme is responsible for the 3α-ol

dehydrogenase activity found in these preparations. A dual nucleotide specificity for the oxidation of androsterone has also been observed with rat liver microsomes (24).

With cortisol as substrate, reversible 11β-ol dehydrogenase activity in human liver resembled that of rat liver in its association with microsomes (25) and its utilization of both DPN and TPN as cofactors (24). Nuclear fractions, which in the rat also contain 11β-ol dehydrogenase activity (26), were not examined in the present study. Human liver microsomes exhibited dehydrogenase activity against 11β-hydroxysteroids having either Δ^4-3-keto or 3α,5β ring A structures in both the C_{19} and C_{21} series. Hübener *et al.* (25), on the other hand, found 11-ketone reduction by rat liver microsomes to occur only with substrates having the Δ^4-3-ketone grouping. Whether this constitutes a species difference or is merely the result of different experimental techniques remains to be established. Bush (27) has pointed out that the occurrence in urine of 11-oxygenated 5β-steroids primarily as 11-ketones and 11-oxygenated 5α-steroids mainly in the 11β-hydroxy form implies some restriction or preferential direction in the interconversion of saturated 11-oxygenated steroid pairs which is dependent upon the stereochemistry of the A/B ring fusion. While the findings of the present study and those of Hübener *et al.* are compatible with this interpretation, neither study is sufficiently extensive to test this hypothesis adequately.

In the studies on reversibility, human liver microsomes reduced 3-ketosteroids to 3β- as well as 3α-hydroxy products. In the presence of oxidized nucleotides, 3β-hydroxy-5α-androstane-17-one, 3β-hydroxy-5β-androstane-17-one and 3β,17,21-trihydroxy-5α-pregnane-11,20-dione were converted to 3-ketosteroids by these preparations. Thus human liver microsomes contain reversible 3β-ol dehydrogenase activity as well as the 3α-ol and 11β-ol dehydrogenase activities which were the major subjects of this study.

Human placenta was found to resemble liver in catalyzing the interconversion of secondary hydroxyl and ketone functions at positions 3 and 11 of corticosteroids and the reduction of the C-20 ketone of such compounds. No indication of 4-ene saturation was obtained in these incubations although Pincus (28) has reported that this may occur in the placenta. Cultured fibroblasts from human uterine tissue have also been reported to be capable of 11β-ol oxidation and 20-ketone reduction of cortisol (29).

As with liver, placental 3α-ol and 11β-ol dehydrogenase activities against a number of steroids were present in microsome particles and required pyridine nucleotides as cofactors. Osinski (30), studying placental 11β-ol dehydrogenase in whole homogenates has also found activity against cortisol, corticosterone and 11β-hydroxy-4-androstene-3,17-dione stimulated by DPN and TPN.

3β-ol Dehydrogenase activity has previously been demonstrated in

human placenta using the 5-ene steroids, dehydroepiandrosterone and pregnenolone, as substrates (31, 32). In the present study, placental microsomes were found to possess such activity and in addition to resemble liver microsomes in exhibiting reversible dehydrogenase activity against saturated 3β-hydroxysteroids.

Little and coworkers (33) have shown the reduction of the 20-ketone group of progesterone to a 20α-hydroxyl by the soluble fraction of human placental homogenates. This reaction required TPNH and was inhibited by microsomes. Since placental microsomes supplemented with TPNH are able to reduce the 20-ketone of tetrahydrocortisone, it seems likely that separate enzymes are responsible for the reduction of these two compounds.

Microsomal fractions from human liver and placenta catalyze the dehydrogenation of a wide variety of hydroxysteroids. The number of individual enzymes responsible for the overall activity of these preparations remains to be established. Attempts at solubilization and physical separation of component enzymes of the microsome complex through the use of acetone, butanol, deoxycholate, digitonin, lipase, phospholipase or trypsin treatment were unsuccessful. However, the finding that cofactor requirements vary with the steroid substrate studied does suggest that a combination of individual enzymes with restricted substrate and cofactor requirements provides the relatively nonspecific capacity of microsomal fractions. In evaluating requirements for exogenous cofactors, pretreatment of microsomes with snake venom containing a pyrophosphatase capable of splitting bound nucleotides has been found an effective means for reducing or eliminating endogenous cofactors not readily removed from microsomes by washing or dialysis. Further study of cofactor-substrate requirements combined with other indirect techniques, such as those employed by McGuire and Tomkins (34) to demonstrate multiple Δ^4-reductase(5α) enzymes in rat liver microsomes, should give a clearer indication of the number and nature of the individual hydroxysteroid dehydrogenase enzymes present in microsome particles.

The overall pattern of corticosteroid metabolism by human tissues demonstrated in the present study is similar in general outline to findings from other species. However, sufficient differences have been revealed in the enzymatic capacities and properties of tissues from different animals to restrict the validity of *in vivo* and *in vitro* correlations without regard to species.

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STUDIES ON THE STATE OF INSULIN IN BLOOD: THE ROLE OF GLUCOSE IN THE *IN VIVO* DISSOCIATION OF INSULIN COMPLEXES[1]

HARRY N. ANTONIADES, KARE GUNDERSEN[2] AND HUGH M. PYLE

Protein Foundation Laboratories, Jamaica Plain, Massachusetts and the Boston Dispensary, and the Department of Medicine, School of Medicine, Tufts University, Boston, Massachusetts

ABSTRACT

The ratio of "free" and "bound" forms of insulin in the sera of non-diabetics has been examined both in the fasting state and following glucose administration. It was found that the predominant form of insulin in the sera of fasting subjects was in the "bound" form. Following I.V. administration of glucose, the "bound" form of insulin disappeared from circulation whereas in ten to twenty minutes after glucose administration the "free" insulin increased markedly.

These studies suggested that a rise of blood glucose in non-diabetics "triggers" a mechanism which results in the dissociation of the "bound" form of insulin in blood.

Preliminary studies in untreated diabetics are also presented and discussed.

THE transport of insulin in blood by other proteins, and more specifically basic proteins, has been described in earlier reports (1–7). It was also shown that a portion of insulin in blood is in the "free" form, the amount depending upon the metabolic state of the individual subject (7). The two forms of insulin in blood, the "free" and the "bound" form, differ in their biological properties in that the "bound" insulin exerts its biological activity only after dissociation of the insulin from its complex (4–7). Dissociation of blood insulin complex(es) has been achieved, *in vitro*, by incubation of serum, or purified blood insulin complexes, with adipose tissue extracts (4, 5, 7).

Studies on the ratio of "bound" and "free" insulin in the sera of non-diabetic subjects showed that this ratio does not represent a purely physicochemical equilibrium between "bound" and "free" insulin (7). It was suggested that the dissociation of "bound" insulin in blood could be affected by other factors, such as those present in adipose tissue extracts.

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The present communication provides evidence that glucose participates in the *in vivo* dissociation of the "bound" insulin in blood. Estimations of the "bound" and "free" insulin in the sera of non-diabetic subjects before and after I.V. administration of glucose indicate that the rise of blood glucose "triggers" a mechanism which results in the dissociation and subsequent utilization of the "bound" form of insulin in blood. Preliminary data on untreated diabetics indicate that the rate of dissociation of the "bound" insulin in blood, following I.V. administration of glucose, may be slower compared to that of non-diabetics.

MATERIALS AND METHODS

Collection of Blood Samples Before and After I.V. Administration of Glucose: Six non-diabetic and two diabetic subjects participated in these studies. Each experiment was begun in the morning, before breakfast, and the participant was fasting overnight. About 100 ml. of blood were drawn from each fasting subject before the glucose administration. These blood samples were marked as samples collected at "0 time." One half gram of glucose per kg. of body weight was injected intravenously into each volunteer subject, and blood samples (about 100 ml.) were collected at various intervals.

The blood samples were allowed to clot, and the sera were separated by centrifugation at 3,000 r.p.m. for thirty minutes at 2° C.

Extraction of "bound" Insulin: Each serum obtained before and after I.V. administration of glucose was passed through a column (3.5×10 cm.) containing 30 to 35 ml. of a cationic exchange resin (Dowex-50×8), in the sodium form, pH 6.4±0.2. This resin is shown to absorb only the "bound" form of insulin (1–7). The resin column was washed with 25 ml. of cold 0.15 M NaCl and the "bound" insulin, absorbed on the resin, was eluted with 25 ml. of 0.1 N NH_4OH followed by washing with 30 ml. of cold distilled water. The ammonium hydroxide eluate and the washes were collected in a beaker containing 4 ml. of 0.2 N H_2SO_4, adjusted to pH 2.0 under continuous stirring. A single glass electrode was immersed in the solution, and the pH was controlled during the elution with the use of a continuous recording pH meter. The final pH was adjusted to 7.0–7.4 and the eluate, plus washes, (50 to 60 ml.) was lyophilized from the frozen state. The dry material was dissolved in 20 ml. of cold distilled water, and the solution was dialyzed twice before the insulin assay, each time against 500 ml. of Gey and Gey bicarbonate buffer.

The resin eluates, representing the "bound" form of insulin in blood, were examined for insulin-like activity by the rat diaphragm assay (8) in the presence of rabbit adipose tissue. Adipose tissue extracts have been shown to dissociate the "bound" insulin *in vitro* (4, 5, 7).

Estimation of "Free" Insulin-Like Activity: The portion of insulin-like activity assayed directly in the sera was considered "free" insulin. This supposition is based on the observation that the "bound" insulin is devoid of activity when examined by the rat diaphragm assay used in this study (8).

Insulin Bioassays were carried out by the diaphragm assay as described by Vallance-Owen and Hurlock (8).

RESULTS

The results are presented in Figures 1–8. The "bound" insulin disappeared from the blood of the non-diabetic subjects 10 to 20 minutes after glucose administration (Figs. 1–6). The "free" form of insulin rose in

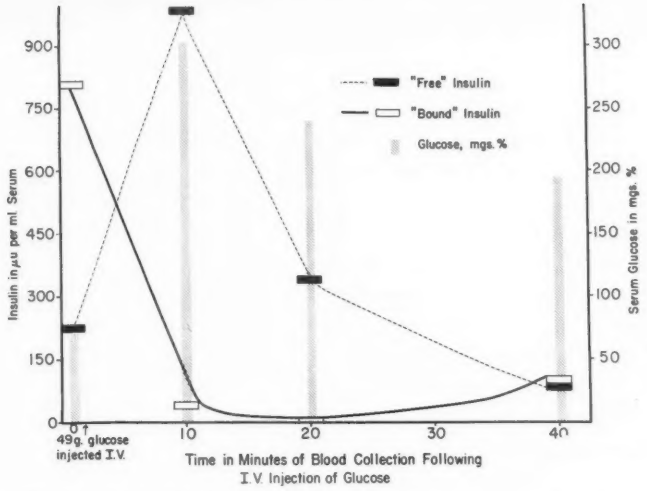

FIG. 1. "Bound" and "free" insulin-like activity in the serum of a non-diabetic subject before and after I.V. administration of glucose.

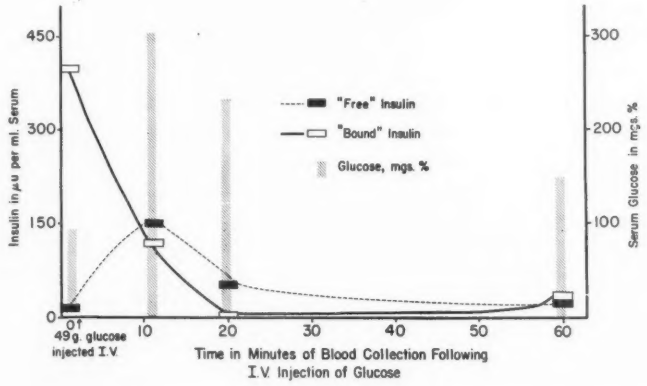

FIG. 2. "Bound" and "free" insulin-like activity in the serum of a non-diabetic subject before and after I.V. administration of glucose.

blood, following glucose administration, achieving a peak about 10 minutes after the injection. The rise of "free" insulin in blood, following glucose administration, is attributed, at least partially, to the dissociation of the "bound" insulin, which is utilized subsequently from the "free" state.

At 40 to 60 minutes after glucose administration, small amounts of "bound" insulin reappeared in blood, and at the end of 4 to 5 hours the ratio of "bound" insulin to "free" again returned to pre-glucose administration levels (Figs. 5, 6).

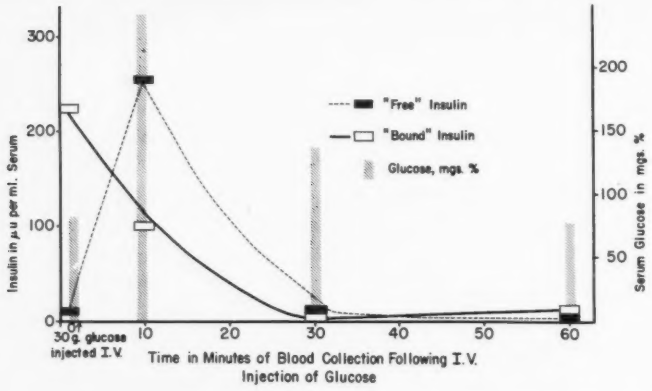

FIG. 3. "Bound" and "free" insulin-like activity in the serum of a non-diabetic subject before and after I.V. administration of glucose.

Preliminary experiments on diabetics are presented in Figures 7 and 8. The rate of dissociation of the "bound" insulin of the untreated diabetic (Figure 7), following glucose administration, is significantly lower compared to that of the non-diabetic subjects. Figure 8 presents the results of a diabetic who discontinued insulin therapy four years ago. Although the "bound" insulin decreases following glucose administration, it does not disappear entirely from circulation. It is interesting to note that in the fasting state (0 time sample) despite an increased serum glucose level (207 mg.%) the predominant form of insulin is the "bound" form.

DISCUSSION

The studies reported in this communication present a link in the chain of events leading to the *in vivo* dissociation and subsequent utilization of the

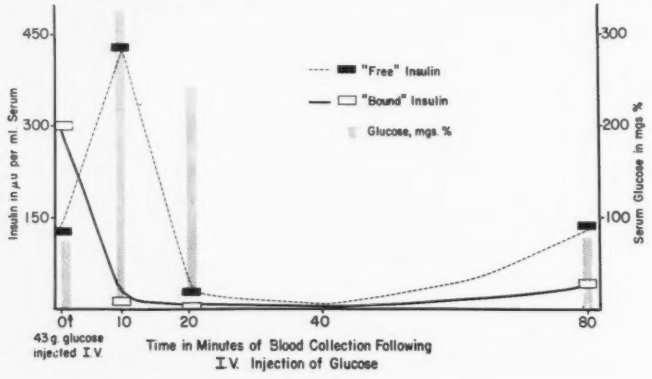

FIG. 4. "Bound" and "free" insulin-like activity in the serum of a non-diabetic subject before and after I.V. administration of glucose.

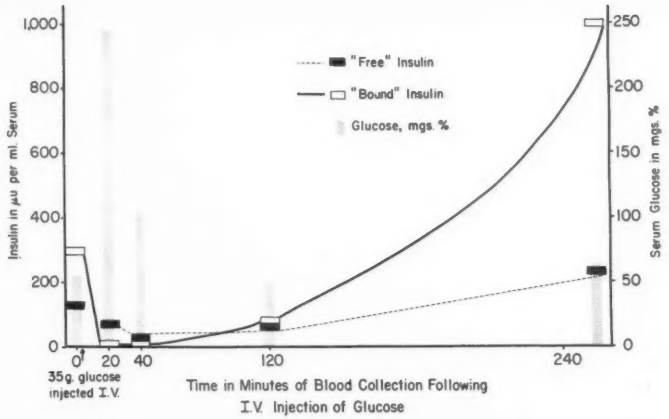

FIG. 5. "Bound" and "free" insulin-like activity in the serum of an untreated diabetic before and after I.V. administration of glucose.

Due to a technical difficulty the 10 minute blood sample was not collected following glucose administration. This sample usually illustrates the increase of "free" insulin in blood after glucose administration.

"bound" insulin in blood. It is shown that a rise of glucose in blood "triggers" a mechanism which increases the rate of dissociation of the blood insulin complex(es). Earlier studies demonstrated that insulin in its "bound" form is devoid of insulin activity when examined *in vitro* by the rat diaphragm assay under specified conditions (4–7). Dissociation of the insulin from its complex was achieved by incubation of the insulin complexes with adipose tissue extracts (4, 5, 7). It was suggested that the "free" insulin in blood represents the biologically active form of insulin in

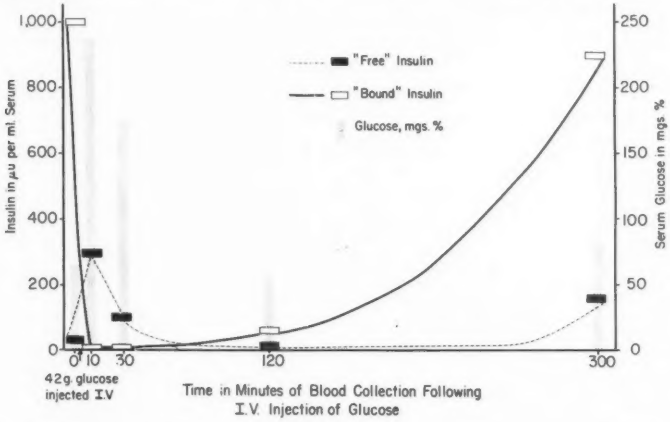

FIG. 6. "Bound" and "free" insulin-like activity in the serum of a diabetic before and after I.V. administration of glucose.

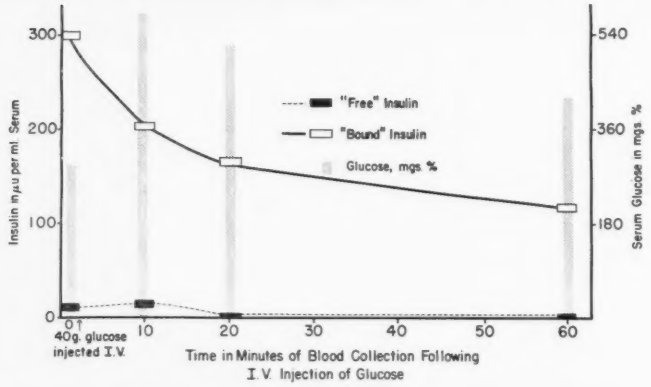

FIG. 7. "Bound" and "free" insulin-like activity in the serum of an untreated diabetic before and after I.V. administration of glucose.

circulation. The "bound" form of insulin was suggested to serve for the transport and storage of insulin in blood in a biologically inactive form (7).

Examination of the ratio of "free" and "bound" insulin in the sera of non-diabetic subjects revealed that this ratio does not result from a pure physico chemical equilibrium (7). It was speculated that the rate of dissociation of the "bound" form of insulin *in vivo* could be affected by other factors possibly similar to those found in the adipose tissue extracts.

The studies presented in this report suggest that a mechanism "triggered" by the blood glucose levels could be responsible for the *in vivo* dissociation of the insulin complexes in blood. It is unlikely that the rise of blood glucose itself results in the dissociation of the insulin complexes.

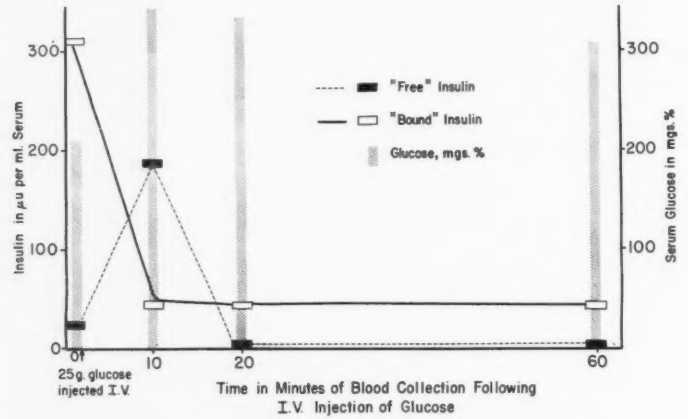

FIG. 8. "Bound" and "free" insulin-like activity in the serum of a diabetic subject before and after I.V. administration of glucose.

Control experiments show that insulin complexes incubated with rat diaphragm in the presence of as much as 300 mg% glucose are devoid of insulin activity in the absence of adipose tissue extracts. The elevation of blood glucose possibly stimulates and activates a mechanism which in turn affects the rate of dissociation of the blood insulin complexes.

The presence of insulin complexes in the blood of diabetic subjects under insulin therapy was described and discussed in an earlier report (4). It was suggested at that time that these diabetics probably could not utilize insulin in its complex form, thus requiring insulin therapy. Preliminary studies in diabetics presented in this communication show the presence of "bound" insulin in their blood. Although the blood glucose levels of these diabetics is elevated, in the fasting state, the predominant form of insulin in their blood is the "bound" form. The rate of dissociation of the "bound" insulin in the untreated diabetic (Fig. 7) is significantly lower, compared to that of the non-diabetic subjects, despite increased glucose levels following glucose administration. The "bound" insulin of the diabetic who discontinued insulin therapy (Fig. 8) decreased 10 minutes after glucose administration but did not disappear from circulation even after 60 minutes. From these preliminary experiments in diabetics one may suggest that some forms of diabetes could be related to a malfunction of the dissociation mechanism of "bound" insulin in blood. Such a malfunction may prevent the utilization of the "bound" form of insulin in those diabetics.

As it was mentioned in a previous report (7) the values for the "bound" insulin should be regarded as the minimum amounts of "bound" insulin in serum. Losses of "bound" insulin may result form the effort to adsorb the insulin complexes on the cationic exchange resin (Na^+ cycle) and in turn to elute and concentrate the individual preparations.

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ON A POSSIBLE ROLE OF β-MELANOCYTE STIMULATING HORMONE (β-MSH) IN THE CENTRAL NERVOUS SYSTEM OF THE MAMMALIA: AN EFFECT OF β-MSH IN THE SPINAL CORD OF THE CAT[1]

WILLIAM A. KRIVOY AND ROGER GUILLEMIN[2]

Department of Pharmacology and Department of Physiology Baylor University College of Medicine, Houston, Texas

ABSTRACT

Intravenous administration of 0.25-2 μg/kg. body weight of highly purified β-MSH increases for long periods of time the amplitude of evoked monosynaptic potentials in the spinal cord of the cat. This is observed in midcollicular decerebrate or barbiturate anesthetized animals. These observations may be a clue to a possible role of β-MSH in the Mammalia as a neurohumor acting in the central nervous system.

THE presence of the Melanocyte Stimulating Hormones, α-MSH and β-MSH, has been well authenticated in the pituitary gland of several genera of Mammalia (1, 2, 3, 4) including the higher primates (5) and man (6, 7, 8). In man, it has been reported that the structure of β-MSH differs from the ovine or porcine material by the addition of 4 amino acids on the N-terminus of the classical β-MSH molecule (7). The physiological significance of these two substances in the mammals is obscure: the role that MSH plays in the mimetic changes of the coloration of teguments and appendages in the lower forms would not appear to have its replicate in the mammals and the multiplicity of metabolic or therapeutic effects reported for "intermedin" or unqualified "melanocyte stimulating hormone" will remain of doubtful value and interest until confirmed with the highly purified peptides α-MSH and β-MSH now available.

Considered together the following information was constructed into a (somewhat thin) working hypothesis proposing that during phylogeny, the melanophoretic hormone might have lost its melanocyto-tropism while retaining or developing neurotropic affinity for the genuinely nervous derivatives of the neural crest, as mimetic processes became less and less important in defensive or adaptive mechanisms: a) melanophores are

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[2] Laboratoire de Morphologie expérimentale et Endocrinologie, Collège de France, Paris.

derived from neural crest (9, 10); b) many drugs (caffeine, lysergic acid derivatives, etc.) or substances of natural origin (serotonin, cathechol amines, acetylecholine, etc.) which reproduce or antagonize the effect of MSH on the melanophores of amphibians (11, and various reviews in 12) are endowed also of some activity in or on the central nervous system; c) melanophoretic activity, allegedly due to MSH, is found in extracts of some parts of the central nervous system (13, 14, 15); d) melatonin, a powerful antagonist of MSH is found throughout the central nervous system (16).

Because of the known structural relationships between α-MSH and ACTH (17) and the recent observations that α-MSH and related peptides (α-MSH analogues) have inherent corticotropic activity (18) and may be converted by pituitary tissue *in vitro* into a material with high ACTH-like activity (Guillemin and Schally, unpublished experiments), it was proposed that our efforts in the present series of experiments should be devoted to a study of possible effects primarily of β-MSH on the central nervous system. A note on some of these experiments has been presented earlier (19).

MATERIALS AND METHODS

Neurophysiology. All experiments were performed on adult cats, of either sex. Early studies were conducted on 6 cats deeply anesthetized with Nembutal; later experiments were conducted on cats decerebrated by midcollicular section, under ether anesthesia (17 animals). The decerebrate preparations were allowed to blow out the anesthetic for at least 2 hours before injection of substances to be studied. The basic technique used here was the electrophysiological method first described by Renshaw (20) for the study of segmental reflex activity of cat spinal cord. This preparation was chosen because of its great sensitivity to pharmacologic agents (21). The technique consists in performing a laminectomy and cutting the dura mater so as to expose the full length of the dorsal and ventral roots subserving the last lumbar spinal segment. The ipsilateral roots are then severed distally. The dorsal root is mounted on a pair of stimulating electrodes and the ventral root on a pair of recording electrodes. Bright silver or chlorided silver electrodes were used indifferently in these experiments. All exposed spinal cord, spinal roots and surrounding muscles are covered with mineral oil previously equilibrated with CO_2 and heated to 37° C. This is accomplished by making a trough from the skin which has been cut in gaining access to the spinal cord and filling the trough with mineral oil. During the course of the experiment the temperature of the mineral oil bath is maintained by radiant heat.

In this preparation, stimulation of a dorsal root gives rise to a ventral root potential which is temporally dispersed as a function of the velocity of nerve fiber conduction and the number of synapses contributing to total synaptic delay time. Therefore by appropriate display of the evoked ventral root potential, one can readily distinguish monosynaptic activity.

Stimulation of the dorsal roots in these experiments was provided by a biphasic stimulus delivered at a frequency of less than 1 c.p.s. (generally 0.5 c.p.s.). The precise frequency used was determined individually for each experiment in order to provide the greatest reproducibility of the ventral root potential. The amplitude and duration of each pulse was adjusted so that the ventral root potential remained between 10% and 50% of

maximal for the monosynaptic potential (typical settings of the stimulator were: 0.54 V., 0.2 msec. duration and 0.5 c.p.s.) Stimulation was continued without interruption for the entire control period and the duration of the experiment. Drugs were administered only after the preparation had remained stable for at least 30 minutes. Stimuli were provided by a Grass Stimulator, (model S4C) isolated from ground by means of a Grass stimulus isolation unit. The ventral root potential was amplified on a Grass (model P5) preamplifier and amplified and displayed on a cathode ray oscilloscope, Tektronix (type 502) or Hewlett Packard (model 130 A). The displayed potential was recorded on photographic paper.

Hormonal Preparations. All substances investigated were injected i.v. through a polyethylene catheter secured in a femoral vein. The several preparations of oxytocin (450 U./mg.), lysine-vasopressin (300 U./mg.), α-MSH (1.5×10^7 U./mg.) and β-MSH (5×10^6 U./mg.) used here were made in this laboratory by A. V. Schally and R. N. Andersen from starting material of hog origin according to methods described elsewhere (22). The assay for melanophoretic activity is the *in vitro* assay of Shizume *et al.* (23) with minor modifications (24); it was used as a 4-point assay with some of the original MSH standard described by Shizume *et al.* (23). The various preparations of purified β-MSH were studied for contamination with other pituitary principles: the maximal contaminations found were: $\leq$1 U. oxytocic activity/mg. β-MSH, 0.1 U. vasopressor activity/mg. β-MSH, when assayed against the U.S.P. Posterior Pituitary Reference Standard. All the experiments reported here were performed with solutions of β-MSH prepared from the lyophilized powder 1 or 2 hours before injection.

In several experiments blood pressure was measured from a femoral artery with a mercury manometer before and after injection of β-MSH. No anticoagulant was used in the system (see below).

RESULTS

Identical results were obtained in both nembutalized and decerebrate cats. In 18 out of 21 experiments in which 2 μg/kg., or more of β-MSH were injected, the monosynaptic potentials were augmented (Fig. 1). This

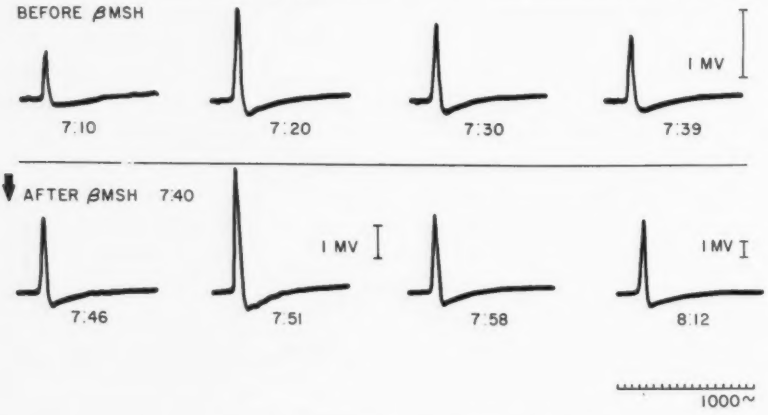

FIG. 1. Excerpts from the photographic records of one experiment. Oscilloscope tracings of evoked monosynaptic potentials in the spinal cord in the cat and calibrations (log-scale of the scope). Decerebrate preparation—numbers under electrical tracings indicate hour of day (P.M.).

enhancement of monosynaptic activity following injection of β-MSH action appeared after a latent period of 4 to 8 minutes and became maximal in about 15–20 minutes, lasting more than one hour. The length of the latent period and duration of effects appeared to be a function of the dose administered. With small doses the latent period was more prolonged and the duration of effects shorter than with large doses. The minimal effective dose of β-MSH required to produce this response was approximately 2 μg/kg. Lower doses generally failed to do so though in one animal, facilitation was observed after injecting 0.25 μg/kg. body weight. Early results indicated that the effects observed on the spinal cord required larger doses of β-MSH when the solutions were prepared 24 hours or more, before the experiment. Hence the decision mentioned above to use only freshly prepared solutions; no specific investigation of this disappearance of activity of the material when in dilute solutions was undertaken in these studies.

Doses of β-MSH as high as 220 μg/kg. failed to produce convulsions or any evidence or irradiation to other spinal segments. Occasionally polysynaptic activity was observed during the height of monosynaptic response. Experiments with decerebrate preparations were undertaken when it was observed that signs of arousal sometimes occurred after injection of β-MSH in the animals under nembutal anesthesia.

When the stimulus applied was maximal, no changes of the evoked potential were observed whatever the dose of β-MSH injected. Also, a stimulus originally capable of evoking a submaximal response was usually found to produce maximal response after administration of β-MSH.

Since vasopressin and oxytocin were known contaminants of the samples of β-MSH used here, vasopressin and oxytocin were administered separately or together in doses equal to or greater than their contamination of the β-MSH. In 13 experiments only one showed an increase in spinal activity of very short duration (2–3 minutes) after vasopressin. In 3 experiments vasopressin and oxytocin were given together in doses corresponding to their respective contamination of 10 μg β-MSH/kg. body weight; there was no increase in the size of the spike potential. Larger doses of these two peptides were not studied here.

Because of the similarity in structure of β-MSH, α-MSH and ACTH, and because of the inherent melanophoretic activity of these last two compounds, the actions of α-MSH and ACTH on the spinal cord were studied. α-MSH was tested in five experiments and ACTH in three. The maximum dose of α-MSH administered was 20 μg/kg.; the maximum dose of ACTH (as highly purified β-corticotropin or corticotropin-A) was 50 μg/kg.; there was no evidence of any action on the spinal cord.[3]

[3] Effects similar to those obtained with β-MSH were observed however in 2 different experiments when *commercial* ACTH preparations were used; this may well be explained by contamination of the corticotropin with β-MSH.

In separate experiments with decerebrate cats, β-MSH had no demonstrable action on blood pressure in the dose ranges which modify activity of the spinal cord. No anticoagulant was used in the recording system after it was found in early experiments that the usual effects of β-MSH on the spinal cord could not be elicited even with large doses of the peptide in an animal given manuronate sulfate in preparations for blood pressure recording.

DISCUSSION

These data show that β-MSH potentiates submaximal evoked potentials in the cat spinal cord. They do not indicate the site of action of the peptide although it appears not to be associated with changes in systemic blood pressure. Within the limits of these experiments the action of β-MSH was not shared by compounds of related structure, thus correcting the original working hypothesis of some correlation between melanophoretic activity and effects on the central nervous system.

The fact that β-MSH never produced convulsions, never changed the evoked potential produced by maximal stimulation (whereas a stimulus originally submaximal was usually found to have become maximal after administration of β-MSH) would suggest that one of the possible roles of β-MSH in the central nervous system is to maintain and modify the central excitatory state of some neuronal systems. A considerable amount of information classical to neurophysiology (25) will have to be available before the suggestion presented above should be considered as more than a tempting hypothesis.

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ON A POSSIBLE ROLE OF β-MELANOCYTE STIMULATING HORMONE (β-MSH) IN THE CENTRAL NERVOUS SYSTEM OF THE MAMMALIA: ENZYMATIC INACTIVATION *IN VITRO* OF β-MSH BY BRAIN TISSUE[1]

JOHN M. LONG, WILLIAM A. KRIVOY AND ROGER GUILLEMIN[2]

Department of Physiology and Department of Pharmacology Baylor University College of Medicine, Houston, Texas

ABSTRACT

Incubation of β-MSH with extracts of brain leads to disappearance of the melanophoretic activity. This effect occurs as a logarithmic function of incubation time, is temperature and pH dependant and seems therefore to be due to an enzymatic inactivation. This effect was not observed in incubating β-MSH with extracts of liver or muscle similarly prepared.

IN ONE of the classics of physiology on the identity of acetylcholine as a chemotransmitter in the nervous system, Chang and Gaddum (1) have set a pattern of the various prerequisites that a substance must meet to be considered as a possible neurohumor endowed with physiological significance. One of these requisites is the presence in the nervous tissue of an enzymatic system which can inactivate the substance.

The experiments reported here were thus a natural sequence to our earlier studies (2), in which a specific effect of β-MSH on the nervous system of the cat had been observed.

MATERIALS AND METHODS

Tissue extracts. Organs (brain, liver, muscle) were obtained from cats killed instantly by cervical dislocation. Tissue homogenates were rapidly prepared with the Potter-Elvejhem glass homogenizer using ice-cold frog Ringer to concentrations of 350–550 mg. fresh tissue/ml. All the procedure was carried in the cold room (0–4° C).

Acetone powders of brain or other tissues were prepared in the cold room (0–4° C) by the usual procedure (ratio of acetone volume/fresh tissue weight = 3/1; all final powders kept in a dessicator over $CaCl_2$ in the cold room). For the experiments reported here, aliquots of the acetone powders (labelled D and Z) were ground in a small mortar with cold frog Ringer (1 ml./10–30 mg. powder); the suspension was then separated in a refrigerated centrifuge at 4° C for 2 hrs. at 5000 r.p.m.; aliquots of the supernatant were

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[2] Laboratoire de Morphologie expérimentale et Endocrinologie, Collège de France, Paris.

used for incubation with equal volumes of solutions of β-MSH in frog Ringer, in various conditions as described below.

Melanocyte Stimulating Hormone. Highly purified β-MSH was prepared in this laboratory by A. V. Schally and R. N. Andersen by a procedure described elsewhere (3). One single batch of β-MSH (AVS-6-38) was used in the experiments reported here; the various dilutions were freshly prepared with frog Ringer for each experiment from frozen aliquots of the original β-MSH stock solution (75 μg/ml. Ringer), stored in sealed pyrex ampoules. The melanophoretic activity of this stock solution did not vary significantly over the period of these studies; in 14 different controls distributed throughout the experiments the specific activity was $0.8 \pm 0.04 \times 10^6$ units/mg.

Method of assay. The melanophoretic activity of the various experimental samples was assessed *in vitro* by a procedure described previously (4) which is essentially the method of Shizume *et al.* (5). The mode of calculation of results in this system and the mathematical characteristics of the performance of this assay as used in our laboratory have been reported (4) and will not be repeated here.

Incubations. They were all carried out in a Dubnoff metabolic incubator in open 20 ml. pyrex beakers, in an air athmosphere. After it was shown in preliminary experiments that incubation of β-MSH with brain extracts would lead to disappearance of the melanophoretic activity, conditions were determined which would consistently produce ca. 50% inactivation. This level of 50% inactivation was considered optimal in view of the limitations of the bioassay with which the melanophoretic activity was followed. With these as standard conditions, effects of changes of pH, temperature, ratio of enzyme/substrate were then studied. At the end of the various incubation periods all solutions were brought to boiling temperature for a few seconds, corrected to original volume when necessary with deionized distilled water and properly diluted for the assay with frog Ringer. For study of pH effects, pH 5.0 was obtained by using an acetate-acetic acid buffer, pH 11.0 by using 0.05 M sodium carbonate. Brain extracts supernatant to be used in control samples were heated for 10 minutes in boiling water at neutral pH. The various experimental protocols, which were classical for this sort of study, are described in the legends and inserts of Figures 1 to 7 and in Table 1. The concentrations of tissue extracts reported refer to weight of the original dry acetone powders used to prepare the extract.

RESULTS

Results obtained with tissue homogenates were similar to those obtained with acetone powders and will not be reported here.

The word *enzyme* when used below and in the legends and inserts of figures has an operational value only standing for brain extract and should not be construed as an implication on our part that we are dealing with a purified enzyme preparation.

Conditions described in Figure 1 were found to produce consistently ca. 50% inactivation of the melanophoretic activity of the β-MSH. These were the standard conditions used to study various factors upon the β-MSH-enzyme system.

In the experiments reported in Figure 2, a curve with *absolute* MSH standards at 2 dose levels was plotted; the results could thus be expressed in absolute activity (MSH units) of the various solutions of in terms of percent MSH inactivated after the various treatments described in the insert. It can be seen that: 1) boiling the enzyme preparation prior to incubation destroys its ability to inactivate β-MSH; 2) heating the β-MSH

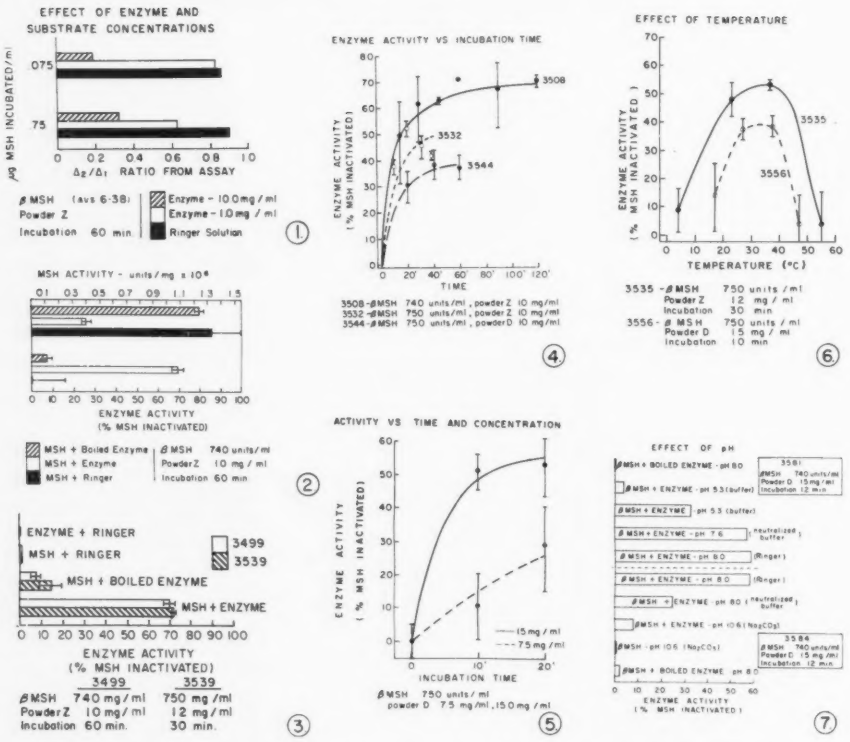
EFFECT OF ENZYME AND SUBSTRATE CONCENTRATIONS
µg MSH INCUBATED/ml
0.75
0.75
0 0.2 0.4 0.6 0.8 1.0
Δ2/Δ1 RATIO FROM ASSAY
β MSH (avs 6-38) Powder Z
Incubation 60 min.
Enzyme - 10.0 mg / ml
Enzyme - 1.0 mg / ml
Ringer Solution
1
ENZYME ACTIVITY VS INCUBATION TIME
ENZYME ACTIVITY (% MSH INACTIVATED)
3508
3532
3544
TIME
3508 - β MSH 740 units/ml, powder Z 10 mg/ml
3532 - β MSH 750 units/ml, powder Z 10 mg/ml
3544 - β MSH 750 units/ml, powder D 10 mg/ml
4
EFFECT OF TEMPERATURE
ENZYME ACTIVITY (% MSH INACTIVATED)
3535
3556
TEMPERATURE (°C)
3535 - β MSH 750 units/ml Powder Z 12 mg/ml Incubation 30 min
3556 - β MSH 750 units/ml Powder D 1.5 mg/ml Incubation 10 min
6
MSH ACTIVITY - units/mg x 10⁴
ENZYME ACTIVITY (% MSH INACTIVATED)
MSH + Boiled Enzyme
MSH + Enzyme
MSH + Ringer
β MSH 740 units/ml Powder Z 10 mg/ml Incubation 60 min
2
ACTIVITY VS TIME AND CONCENTRATION
ENZYME ACTIVITY (% MSH INACTIVATED)
1.5 mg/ml
7.5 mg/ml
INCUBATION TIME
β MSH 750 units/ml powder D 7.5 mg/ml, 1.5 mg/ml
5
EFFECT OF pH
3581
β MSH + BOILED ENZYME - pH 8.0
β MSH 740 units/ml Powder D 1.5 mg/ml Incubation 12 min
β MSH + ENZYME - pH 5.3 (buffer)
β MSH + ENZYME - pH 5.3 (buffer)
β MSH + ENZYME - pH 7.6 (neutralized buffer)
β MSH + ENZYME - pH 8.0 (Ringer)
β MSH + ENZYME - pH 8.0 (Ringer)
β MSH + ENZYME - pH 8.0 (neutralized buffer)
β MSH + ENZYME - pH 10.6 (Na2CO3)
3584
β MSH - pH 10.6 (Na2CO3)
β MSH 740 units/ml Powder D 1.5 mg/ml Incubation 12 min
β MSH + BOILED ENZYME pH 8.0
ENZYME ACTIVITY (% MSH INACTIVATED)
7
ENZYME + RINGER
MSH + RINGER
MSH + BOILED ENZYME
MSH + ENZYME
3499
3539
ENZYME ACTIVITY (% MSH INACTIVATED)
3499 3539
β MSH 740 mg/ml 750 mg/ml
Powder Z 10 mg/ml 12 mg/ml
Incubation 60 min. 30 min.
3

FIG. 1. Effect of enzyme/substrate concentration. For calculation and significance of $\Delta 2/\Delta 1$ (see Reference No. 4 and 5).

FIG. 2. Inactivation of the enzymatic activity of the brain extract by heating in boiling water. Above, results calculated in absolute values (MSH u./mg.); below, as percent of MSH inactivated.

FIG. 3. Two different experiments (3499; 3539) on inactivation of the enzyme by heat; the brain extract has no direct melanophoretic or antimelanophoretic effects.

FIG. 4. Enzymatic activity vs. incubation time. Three different experiments (3508, 3532, 3544).

FIG. 5. Enzymatic activity as a function of incubation time and concentration of the brain extract.

FIG. 6. Effect of temperature on the enzymatic activity of the brain extract. Two experiments (3535, 3556) with two different concentrations of the enzyme.

FIG. 7. Effect of various pH on the enzymatic activity of the brain extract. Two experiments (3581, 3584).

TABLE 1

Tissue extract	Melanophoretic activity ($\Delta 2/\Delta 1$)*	Enzymatic activity % MSH inactivated
Brain	.43 ± .12	50
Liver	.82 ± .06	0
Muscle	.96 ± .11	0
Ringer	.81 ± .08	0

Incubation of β-MSH (750 U./ml.) with various tissues extracts (10 mg./ml.) at 37° C for 30 minutes.

* See reference 4 for computation and significance of $\Delta 2/\Delta 1$.

solution in Ringer for 1-5 minutes in boiling water does not alter the melanophoretic activity. Figure 3 shows similar experiments with essentially similar results. It shows further that the brain extract has no inherent melanophoretic activity. Figure 4 shows the results of 3 different experiments in which duration of incubation time was studied at 37° C, other conditions as described in the insert. In all 3 experiments, the inactivation of β-MSH occurs in a logarithmic fashion, the greater part of the reaction taking place within the first minutes of incubation. Figure 5 reports the effects of two different concentrations of the brain extract on a constant amount of β-MSH, at two different incubation times. Figure 6 shows that optimal activity of two different brain extracts in two different experiments was between 30–40° C and fell off sharply outside of this range. The optimal pH to observe inactivation of β-MSH by the brain extract appears to be close to neutrality as shown in Figure 7; this figure shows also that results were not due to alterations of either β-MSH or response of the assay system by changes in pH.

In conditions similar to those used for brain extracts, incubation of β-MSH with extracts of liver or muscle led to no disappearance of the melanophoretic activity (Table 1).

Effects of incubation with brain extracts on the neurotropic activity of the β-MSH were not investigated further in this series of experiments when it was shown that intravenous injection of the tissue extracts to the cat would lead to immediate decrease of the recorded electrical activities of the spinal cord preparation; this was accompanied by sudden fall in the blood pressure of the animal.

In one experiment the brain extract (10 mg./ml.) was shown to inactivate the melanophoretic activity of a solution of α-MSH (3.75 μg, 1000 U./ml.) by 75%.

DISCUSSION

The effects reported here are best explained by the presence in brain extracts of some enzyme system which is able to inactivate β-MSH. It was not intended in the present series of experiments to further purify and characterize this enzymatic system. Thus we have no knowledge about its

(chemical) specificity, a problem which does not necessarily have any bearing on the physiological significance of the effects observed. Similarly we have no data on pharmacological drugs as possible specific inhibitors of this enzymatic inactivation. It is of interest however that effects observed were not seen with tissues other than brain, an observation which would also tend to eliminate blood as the source of this enzymatic activity. In earlier studies, Shizume and Irie (6) reported significant inactivation of a crude MSH preparation by homogenates of liver, kidney and muscle from dog origin, and less than 10% inactivation by homogenates of brain. It is difficult to compare their results with ours as the substrate/enzyme ratios in their experiments were vastly different from those used here. When conditions similar to theirs were repeated by us, their results were indeed confirmed; however the larger quantities of β-MSH relative to tissue extracts in the incubation system, as in the experimental conditions described here, led consistently to the results reported in this paper. It may also be difficult to compare the results obtained here with the highly purified peptide to those of Shizume and Irie using a preparation considerably less purified (1.4×10^4 units/mg.).

At this point it is of interest to confront our present knowledge of β-MSH with the necessary requirements for a substance to be considered as a neurohumor of possible physiological significance. These include:

a. *Identification of the material in the tissue area where it is thought to act.* Several reports have described presence of melanophoretic activity (allegedly due to MSH) in extracts of various areas of the central nervous system (see 2). Recently, our group has specifically characterized the presence of β-MSH in the hypothalamus of a mammal (hog) by its chromatographic mobility on an ion exchanger (7) and on electrophoresis.

b. *A specific physiological effect must be directly attributed to the substance.* Demonstration of the increase in amplitude of the monosynaptic spinal potentials (2) after injection of minute quantities of β-MSH shows an action of the peptide upon the central nervous system, which was not shared by α-MSH, β-ACTH, oxytocin or vasopressin.

c. *An enzyme system capable of inactivating the substance at its (likely) site of action.* The results reported here are in agreement with a similar explanation.

d. *There should exist a specific antagonist to the action of the substance.* Lerner *et al.* (8) have isolated melatonin, a potent antagonist of the melanophoretic activity of MSH. The substance is found in pineal extracts, throughout the central nervous system and also in peripheral nerves. Nothing is known, at this time, about possible antagonism of melatonin to the neurotropic effects of β-MSH.

These considerations, those developed in the preceding paper as well as the recent observations of Wright and Lerner (9) are in agreement with the concept that β-MSH may be a neurohumor. It must be remem-

bered however, that much of this information relates to the melanophoretic activity of β-MSH; we have seen (2) that there is no necessary correlation between melanophoretic activity and neurotropic activity as α-MSH does not affect the monosynaptic potentials of the spinal cord. Much of the future studies on this project proposing to understand the significance of β-MSH in the mammals will therefore have to demonstrate further, specific effects of the peptide in the nervous system.

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NOTES AND COMMENTS

THE BEHAVIOR OF KIDNEY MITOCHONDRIA UNDER THE INFLUENCE OF ADH

ABSTRACT

Mitochondria were isolated by ultracentrifugation from homogenates of both medullary and cortical areas of dog kidney. The extent of particle swelling was estimated by optical methods in sucrose solutions. Mitochondria isolated from medullary areas had a much higher content of osmotically active material than did those from cortical areas as determined by the molarity of sucrose in which swelling failed to occur. Vasopressin caused an increased uptake of water in medullary mitochondria as measured by this method but had no effect on cortical mitochondria.

The Wirz (1) countercurrent theory to explain the concentrating activity of the kidney tubule has been widely accepted. A portion of the theory demands that the medullary cells in the collecting ducts and/or the Loops of Henle be hypertonic with respect to the plasma and to the cortical cells. It has been postulated that ADH acts upon this system to "open pores" in the tubules so that water can be transferred from the lumen to the interstitial spaces by some intercellular or intracellular process. The simple experiments described below suggest that ADH may act within the cells.

METHODS

Kidneys were removed within seconds after the induction of Nembutal anesthesia in dogs averaging about 10 kg. body weight. The kidneys were placed on ice, rapidly split in a sagital plane and the cortical and medullary tissue was removed with iris scissors. The two portions were rapidly homogenized with a Potter-Elvejhen homogenizer in 0.44 M sucrose containing 0.02% Heparin and centrifuged twice at $1200 \times g \times 10'$ in the Spinco Model L ultracentrifuge to remove nuclei and cellular debris. The supernatant was then centrifuged at $10{,}000 \times g \times 10'$ twice to sediment the mitochondria. Samples of the mitochondrial pellet were taken to determine the extent of contamination with other particles (2). Almost pure preparations of mitochondria were obtained.

The pellet was homogenized gently in 0.44 M sucrose and diluted to give an optical density of 0.600 (approx.) at 520 millimicrons in the 1 cm. cells of the Beckman DU spectrophotometer. The optical density of the mitochondrial suspensions were compared on the same original preparation at the same time and over a 30 minute period with and without the addition of 10 μg of lysine-vasopressin (ADH).[1] Some preliminary attempts were made to determine the osmolarity of the mitochondrial contents by estimating swelling in various concentrations of sucrose.

RESULTS

Results presented in Figure 1 are typical of several similar experiments. Two observations are immediately apparent. The cortical mitochondria do not take up water from a 0.44 M solution indicating that this solution is almost isotonic with the particle. In 0.25 M sucrose the particles swell rapidly as indicated by a rapid decrease in optical density, and in 0.60 M sucrose the mitochondria increase in optical density indicating a loss of compartment water. In distinct contrast the medullary mitochondria take up water from a 0.44 M sucrose solution indicating a hypertonicity of the mitochondrial content and the concentration of sucrose must be raised to about 0.60 M before they fail to take up water. The addition of ADH produces widely varying results between the two mito-

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[1] The lysine vasopressin was kindly provided by Drs. Schally and Guillemin, Baylor University School of Medicine, Houston, Texas.

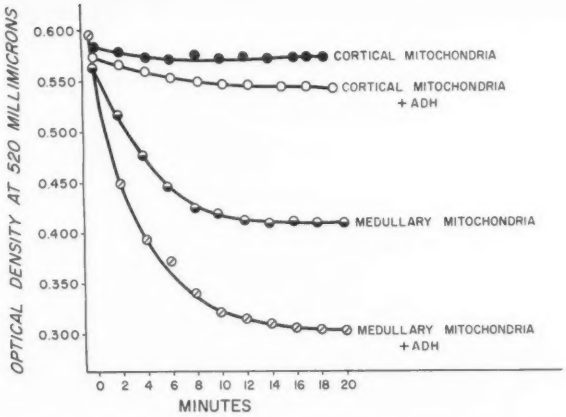

FIG. 1. The typical response of kidney and medullary mitochondria in 0.44 M sucrose to 10 μg of lysine vasopressin added in 0.1 ml. sucrose to 3 ml. of suspension. The ADH was prepared fresh for each experiment by suspending the desired amount in the unbuffered sucrose solution used for the experiment.

chondrial suspensions. ADH has almost no effect on the cortical mitochondria. On the other hand, the uptake of water by the medullary particles is markedly accelerated in the presence of ADH and the final equilibrium reached appears to be at a point where the water content of the particles is greater than at the normal equilibrium point.

These preliminary results do not permit generalizations about the action of ADH on water transfer in cells but they do suggest that ADH may in some manner facilitate water movement. If such were the case, it would be expected that the mitochondria which behave as osmometers (3) would be provided with some means of regulating their water transfer to that of the cell as a whole. Otherwise, we can imagine the mitochondria expanding and contracting within the kidney cell with each alteration of water balance in the organism. It should be emphasized that the mitochondria may not behave as *perfect* osmometers but in this case mitochondria from different sections of the kidney were compared in identical solutions so that differences should be due to the living material rather than the suspending media. These results appear to be confined to the kidney as liver and pituitary mitochondria show no change in water balance as measured by changes in optical density when they are exposed to ADH.

J. H. U. BROWN AND ATALANTE A. PETKAS

Division of General Medical Sciences
National Institutes of Health
Bethesda, Maryland

Emory University
Atlanta, Georgia

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ADRENAL CORTICOSTERONE IN C MICE FOLLOWING OVARIECTOMY[1]

ABSTRACT

During the third month after ovariectomy there is a consistent lowering of corticosterone content in adrenals of C (Bagg albino) mice, whether the operation is done early during the second month of life or at three months of age. This effect of ovariectomy upon adrenal corticosterone content is readily demonstrable under conditions standardized for periodicity analysis, but it may be obscured if standardization procedures are omitted.

Adrenal corticosterone content determined fluorometrically (1) is higher in female C mice than in C males (2). If an ovarian-adrenal interaction contributes to such findings, it can be expected—and it was found—that the corticosterone content of the female adrenal is lowered by ovariectomy.

METHODS

C mice were used for study. This stock had been maintained by brother-to-sister mating for over 18 years in the Division of Cancer Biology, Department of Pathology, at the University of Minnesota. The animals were ovariectomized by a dorso-lateral approach under ether anesthesia at the ages indicated in Table 1. The upper third of the uterine tube was removed with the ovary and the paraovarian fat in a one-stage procedure. Vaginal smears on material collected by lavage from 10 sham-operated and 10 ovariectomized mice during the second month after operation were done between 1600 and 1700, Monday to Friday, for 2 weeks.

TABLE 1. EFFECT OF OVARIECTOMY UPON ADRENAL CORTICOSTERONE IN C MICE[1]

Exp. No.	Age (days) at oper. (approx.)	Days post-op. (approx.)	Oper. type (X)	N. of determ. (Y)	Content μg/ gland pair X ± 1 S.E.	Difference X ± 1 S.E.	P
			Standardized conditions[2]				
I	37	62	O	5	.2588 ± .0307	.1065 ± .0437	*
			S	5	.3653 ± .0311		
II	37	83	O	5	.2203 ± .0160	.1707 ± .0558	*
			S	5	.3910 ± .0535		
III	84	71	O	6	.2062 ± .0201	.1583 ± .0344	**
			S	6	.3645 ± .0266		
			Routine conditions (natural lighting)				
IV	65	62	O	7	.2029 ± .0114	.0350 ± .0379	
			S	7	.2379 ± .0361		

[1] Sacrifice at presumed peak time of rhythm, midpoint about 1600.
[2] For light-synchronized periodicity analysis (3) (light from 0600 to 1800); (X) O = ovariectomy, S = sham operation; (Y) Each determination on at least 4 adrenals.
* P significant below 5%.
** P significant below 1%.

Cornified vaginal epithelium was found only in sham-operated mice. Completeness of ovariectomy also was examined at autopsy. Gross motor activity of 5 ovariectomized and 5 sham-operated mice also was studied in special actometers and its synchronization with a 24-hr. light-dark schedule was ascertained for both groups.

Groups of animals were sacrificed at several post-operative time points, Table 1. Prior to sacrifice, some groups, but not all of them, were kept under circumstances standardized for periodicity analysis (3). Whether or not conditions were standardized, the mice were sacrificed consistently between 1500 and 1700, i.e., at the time of the anticipated peak of corticosterone rhythm (4–8). Routine conditions, Table 1, refer to multiple housing, natural lighting, and room temperature changing slightly with the seasons.

The adrenals removed with procedures described elsewhere (4) were immediately frozen in dry ice and corticosterone was determined by the fluorometric procedure of Silber *et al.* (1).

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The usefulness of this relatively simple procedure for periodicity anlaysis and its limitation with respect to specificity have been discussed elsewhere (6). Corticosterone contents are presented in absolute terms but serve solely for inter-group comparisons.

RESULTS AND DISCUSSION

Three experiments done under conditions standardized for periodicity analysis, Table 1, show a significant lowering of adrenal corticosterone content at several months following ovariectomy. In an additional study (routine conditions, experiment IV) the inter-group difference in corticosterone content was of the same sign as in the standardized experiments, but it was not statistically significant. It seems likely that the rhythms of corticosterone in different individuals constituting a given group were less closely synchronized in phase under routine conditions; hence, a greater variability in both groups had obscured the significance of the inter-group difference.

Moreover, in much additional work done by some of us with others on D_8 (Dilute Brown, subline 8), B_1 (C_{57} Black, subline 1) as well as C mice, corticosterone contents were invariably lower in ovariectomized mice than in sham-operated controls and these differences were significant so long as environmental conditions were controlled.

These results have a bearing, *inter alia*, upon a model of breast cancer in the mouse induced by heterotopic pituitary isografts (2, 9–11). Following the latter procedure, a rise in adrenal corticosterone was found in C-stock females during a period from the third to the sixth month post-implantation (2). Herein, the higher adrenal corticosterone content of female C mice as compared to males was seen to be, at least in part, a function of the ovary. Whether elevations of adrenal corticosterone content after pituitary isografting (2) also involve ovarian function requires further study, but preliminary data suggest that this graft effect is significant in ovariectomized C-mice.

FRANZ HALBERG, JOHN J. BITTNER, HARVEY L. COLE,
ERHARD HAUS AND IRWIN KAISER

Division of Cancer Biology
Department of Pathology
University of Minnesota, Minneapolis
and
Department of Obstetrics and Gynecology
University of Utah Medical School
Salt Lake City, Utah

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LONG-TERM EFFECTS OF MUSCULAR WORK ON THE ADRENAL MEDULLA OF THE MOUSE[1]

ABSTRACT

Albino mice were allowed to swim till exhaustion daily for 30 days. The volume of the adrenal medulla and the relative amounts of adrenaline- and noradrenaline-containing tissues were measured planimetrically. Swimming caused an increase in the volume of the noradrenaline-containing medullary parenchyma without affecting the volume of the adrenaline-storing parenchyma. This change was reversible by one month's rest.

In the adrenal medulla of several mammalian species, including the mouse, there are two types of parenchymal cells, one storing mainly adrenaline, the other noradrenaline (1–4). As far as we know, the effect of long-term muscular work on the two types of adrenomedullary cells has not been investigated earlier. In view of the importance of this type of stimulus such a study was performed and the results are reported in the present paper.

MATERIAL AND METHODS

The material consisted of 55 albino mice. Of these, 16 served as controls, while the remaining 39 mice were allowed to swim in warm (37° C) water daily (excepting Sundays) till exhaustion. This took about 40 min. during the first few days, but increased with training up to 4 hours during the last (4th) week. The total length of the swimming period was 30 days. Since mice are able to float on water without moving, lead weights were attached on their tails. In each swimming session they were rescued just before submerging. However, 25 mice drowned and only 14 mice survived the 30 days period. Seven of these were killed 24 hours after the last swimming day together with 7 untreated controls. The remaining 7 swimmers were allowed to rest for a month; they were then killed together with 9 untreated controls.

The left adrenals were fixed in a dichromate-formalin mixture, sectioned serially, and the volume of the medulla was measured planimetrically (5). Right adrenals were treated for the iodate reaction (4) and the relative volume of the iodate-positive (noradrenaline-containing) medullary tissue was estimated planimetrically from 4 mid-sections of each gland.

RESULTS

During the one month's swimming period, the experimental mice gained in weight less than the controls. During the resting period of another month the body weight returned to the same level as that of the controls.

Table 1 shows the effect on the adrenal medulla. The absolute volume of the medulla did not differ significantly from that of the corresponding control group in either of the experimental groups. However, when the medullary volume was expressed as mm.^{3} per kg. of body weight, there was an increase of about 20% in the mean volume of the medulla in the group killed immediately after swimming. This change was statistically significant at the level $P \sim 0.03$.

Both the relative proportion and the absolute volume of the iodate-positive tissue increased significantly with about 40% during swimming (Table 1). When calculated in terms of body weight (not shown in the table), the increase was even greater, about 75% and more significant ($P \sim 0.000{,}2$) than the change in the absolute volume, shown in the table. No significant changes were observed in the volume of the iodate-negative tissue, whether this was expressed as mm.^{3} or in terms of body weight.

In addition to the volume increase in the iodate-positive tissue of the mice killed after swimming, an increased intensity of the iodate reaction was observed in the specific medullary islets. This suggests that there was also an increase in the noradrenaline concentration in the cytoplasm of these cells.

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[1] Work supported by research grants from Suomen Kulttuurirahasto (Finland's Cultural Fund) and the National Institute of Arthritis and Metabolic Diseases, U. S. Public Health Service (A-1725).

TABLE 1. EFFECT OF SWIMMING ON THE ADRENAL MEDULLA OF THE MOUSE

Treatment	Medullary volume (mm.³)		Iodate-positive tissue (%)		Iodate-positive tissue (mm.³)		Iodate-negative tissue (mm.³)	
Controls (7)[1]	0.323[2]	0.070[3]	15.2	4.1	0.048	0.012	0.276	0.067
Swimming for 30 days (7)	0.291	0.042	21.7	2.4	0.063	0.009	0.228	0.036
Significance of difference (P)[4]	—		0.004		0.02		—	
Controls (9)	0.516	0.117	15.3	4.1	0.078	0.020	0.438	0.109
Swimming for 30 days, rest for 30 days (7)	0.414	0.079	14.1	4.0	0.061	0.024	0.353	0.064
Significance of difference (P)	—		—		—		—	

[1] Number of animals.
[2] Mean.
[3] Standard deviation.
[4] Level of significance of difference from the control group, calculated with the t-test.

There were no significant differences between any of the variables measured between the swimming group killed after one month's rest and that of the control group.

The results described are different from those obtained in a parallel study on rats (6), in whose adrenals the volume of both the adrenaline- and the noradrenaline-storing parts of the medulla increased. While this difference may in part be due to differences in the experimental set up, it is more likely that the mode of response of the adrenal medulla of the mouse is different from that of the rat (7, 8).

O. ERÄNKÖ AND M. HÄRKÖNEN

Department of Anatomy
University of Helsinki
Siltavuorenpenger
Helsinki, Finland

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GROWTH HORMONE AND FATTY ACID MOBILIZATION: THE ROLE OF THE PITUITARY, ADRENAL AND THYROID[1]

ABSTRACT

Growth hormone induces a significant increase in plasma free fatty acid (FFA) concentration when administered to hypophysectomized, adrenalectomized, and thyroidectomized as well as to normal rats. The magnitude of the growth hormone effect is reduced in adrenalectomized but not in adrenal-demedullated, thyroidectomized or hypophysectomized animals.

It is now firmly established that growth hormone administration results in the mobilization of nonesterified or free fatty acids (FFA) from adipose tissue as evidenced by increased plasma FFA concentrations (1, 2, 3) and increased release of FFA from adipose tissue *in vitro* (4). The following study was undertaken to investigate the influence of the pituitary, adrenal and thyroid glands on the fatty acid mobilizing action of growth hormone.

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[1] Supported by a grant A-3754 from the U.S.P.H.S.

MATERIALS AND METHODS

Mature female rats of the Holtzman and Charles River strains were used in this study. They were given Purina lab chow and water *ad libitum* until the beginning of the experimental period. Adrenalectomized rats were provided with 0.9% saline in addition to tap water and were studied one week post-operatively. Saline was also available to adrenal-demedullated rats for the first post-operative week. Experimentation on these animals was begun 2 weeks after surgery. Thyroidectomies were performed 2 months prior to experimentation. Hypophysectomized rats were obtained from the Charles River Breeding Laboratories and were studied 2 weeks post-operatively. These animals received no dietary supplements during this period. The completeness of hypophysectomy was verified by microscopic inspection of the *sella turcica* at autopsy.

At the beginning of each experiment, all rats were subjected to a 12-hour fast following which 1 mg. of porcine[2] or ovine[3] growth hormone was administered intraperitoneally in 1 ml. of water adjusted to pH 8–9. Control animals were injected with saline. Six hours later, after a total fasting period of 18 hours, the rats were anesthetized with ether, and blood for FFA analyses was collected from the abdominal aorta into heparinized syringes. FFA determinations were made in duplicate on 1 ml. aliquots of plasma by the method of Dole (5).

TABLE 1. THE EFFECT OF GROWTH HORMONE ON PLASMA FFA CONCENTRATION IN NORMAL, ADRENALECTOMIZED, ADRENAL-DEMEDULLATED, HYPOPHYSECTOMIZED AND THYROIDECTOMIZED RATS

Animals	No. of rats	Plasma FFA μEq/ml.	Increment μEq/ml.	P
Normal				
Saline	9	0.64 ± 0.03*		
Porcine G. H.†	12	0.91 ± 0.04	+0.27	<0.01
Saline	6	0.66 ± 0.07		
Ovine G. H.	6	1.04 ± 0.09	+0.38	<0.01
Adrenalectomized				
Saline	8	0.44 ± 0.05		
Porcine G. H.	7	0.60 ± 0.04	+0.16	<0.02
Adrenal-demedullated				
Saline	6	0.63 ± 0.04		
Ovine G. H.	6	0.98 ± 0.10	+0.35	<0.01
Hypophysectomized				
Saline	6	0.43 ± 0.03		
Porcine G. H.	6	0.74 ± 0.06	+0.31	<0.01
Thyroidectomized				
Saline	10	0.56 ± 0.04		
Porcine G. H.	10	0.82 ± 0.08	+0.26	<0.02

* Mean ± Standard Error.
† All growth hormone injections given I.P. 6 hours prior to sacrifice (1 mg./rat).

RESULTS

Growth hormone administration causes a significant increase in plasma FFA concentration in hypophysectomized, adrenalectomized, adrenal-demedullated and thyroidectomized rats (Table 1). However, the magnitude of this growth hormone effect is reduced in adrenalectomized rats, although the normal response to growth hormone administration occurs in the other groups. The fasting (control) FAA levels tend to be reduced in adrenalectomized, hypophysectomized and thyroidectomized rats, but not in adrenal-demedullated animals (Table 1).

DISCUSSION

The lipid mobilizing effect of growth hormone and anterior pituitary extracts (see (for review) has been found by some to be reduced or abolished in adrenalectomized rat

[2] Prepared and generously supplied by Dr. M. S. Raben.
[3] Gift of the Endocrinology Study Section, U.S.P.H.S.

as judged by changes in concentration of hepatic lipids (7, 8, 9) and blood and urine ketones (9, 10, 11) but others could not demonstrate an effect of adrenalectomy on this action of growth hormone (12, 13, 14, 15). The present finding that the stimulatory action of growth hormone on FFA mobilization is reduced by adrenalectomy lends support to the view that the adrenal cortex plays a permissive role in this response. Since adrenal-demedullation does not influence FFA mobilization following growth hormone administration, it is clear that the absence of the adrenal cortex rather than the medulla accounts for the reduced response of adrenalectomized rats.

The observation that hypophysectomy does not interfere with the FFA mobilizing action of growth hormone is in agreement with previous findings in hypophysectomized monkeys (3) and rats (2). In addition, hepatic fat accumulation (16) and ketonemia (14) occur in hypophysectomized rats injected with growth hormone or anterior pituitary extract. Similarly, the finding that the absence of the thyroid does not influence FFA mobilization in growth hormone treated rats is in accord with earlier reports that, in the rat, thyroidectomy does not modify the ketogenic action of anterior pituitary extracts (9, 14) although in rabbits (17) and dogs (18) injected with such extracts the ketonuria normally observed is abolished by thyroidectomy.

The data presented in this communication support the conclusion that growth hormone can exert its lipid mobilizing action in the absence of the pituitary, thyroid and adrenal glands, although in the latter case the response to growth hormone is reduced.

H. M. GOODMAN[4] AND E. KNOBIL

Department of Physiology
Harvard Medical School
Boston, Massachusetts

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[4] Predoctoral Research Fellow of the National Cancer Institute, U.S.P.H.S.; present address: New England Center Hospital, Boston, Mass.

[5] Present address: Department of Physiology School of Medicine, University of Pittsburgh, Pittsburgh 13, Pa.

THE ENDOCRINE SOCIETY

1962 AWARDS

Fred Conrad Koch Award

In 1957 a substantial legacy was bequeathed to the Society by the late Elizabeth Koch for the purpose of establishing the Fred Conrad Koch Memorial Fund in memory of her late husband, distinguished service professor of physiological chemistry at the University of Chicago and pioneer in the isolation of the androgens. This is to be the highest honor of the Endocrine Society and is to be represented by a medal that is to be known as the Koch Medal of The Endocrine Society. The medal and honorarium of $3,500 is to be given annually to an individual for work of special distinction in endocrinology.

The recipient shall be chosen from nominations presented by members of the Society and is limited to citizens of the United States and Canada. This award will replace the Medal of the Endocrine Society which was established in 1954 and presented to Dr. Carl Moore in 1955, Dr. Frederick L. Hisaw in 1956, Dr. Joseph C. Aub in 1957, Dr. I. L. Chaikoff in 1958, Dr. Wilbur W. Swingle in 1959, Dr. Emil Witschi in 1960 and Dr. Lawson Wilkins in 1961. The Endocrine Medal replaced the E. R. Squibb Award which was formerly the highest honor bestowed by the Society. Past recipients of the Squibb Award were Dr. George W. Corner in 1940, Dr. Philip E. Smith in 1941, Dr. Fred C. Koch, 1942, no award was given in 1943, Dr. E. A. Doisy in 1944, Dr. E. C. Kendall in 1945, Dr. Carl O. Hartman in 1946, Drs. Carl F. and Gerty T. Cori in 1947, Dr. Fuller Albright in 1948, Dr. Herbert Evans in 1949, Dr. C. N. H. Long in 1950, Dr. J. B. Collip in 1951, Dr. James H. Means in 1952 and Dr. David Marine in 1953.

The Ciba Award

The Ciba Award, to recognize the meritorious accomplishments of an investigator who has not attained his 36th birthday by June 1st of the year the award is presented, in the field of clinical or preclinical endocrinology, was established in 1942, but no recipient was selected in 1942 or 1943. In 1944 the Award was presented to Dr. E. B. Astwood, 1945 Dr. Jane A. Russell, 1946 Dr. Martin M. Hoffman, 1947 Dr. Choh Hao Li, 1948 Dr. Carl Heller, 1949 Dr. George Sayers, 1950 Dr. Oscar M. Hechter, 1951 Dr. Albert Segaloff, 1952 Dr. Seymour Lieberman, 1953 Dr. Sidney Roberts and Dr. Clara Szego (Mrs. Roberts), 1954 Dr. Isadore M. Rosenberg, 1955 Dr. Jack Gross, 1956 Dr. Alfred M. Bongiovanni, 1957 Dr. Nicholas S. Halmi, 1958 Dr. Monte Arnold Greer, 1959 Dr. Gordon L. Farrell, 1960 Dr. Don H. Nelson and 1961 Dr. Ernst Knobil. Prior to 1952 the Award was $1,200. It has now been increased to $2,500.

The Ayerst and the Squibb Fellowships

The Ayerst Fellowship was established in 1947 and the Squibb Fellowship in 1956. They are designed to assist men or women of exceptional promise in furthering their advancement towards a career in endocrinology. Each Fellowship is awarded on alternate years and the stipend, which

will not exceed $7,500 may be divided into two Fellowships in varying amounts in accordance with the qualifications of the appointees. Individuals possessing the M.D. or Ph.D. degree, or candidates for either of these degrees, are eligible for appointment.

Applicants must submit the following information:

1. Evidence of scientific ability as attested by studies completed or in progress.
2. Recommendations from individuals familiar with the candidate and his work.
3. A proposed program of study.
4. Acceptance of the individual by the head of the department in which the Fellowship will be held.
5. A statement that he or she will serve full time if awarded a Fellowship. A small amount of time (10 to 15 per cent) may be spent in course work or participation in teaching, the latter purely on a voluntary basis.

The Upjohn Scholar of The Endocrine Society

The Council of The Endocrine Society has established a category of Scholars. These grants have been made available through the generosity of the Upjohn Company, and will be awarded to an established investigator or teacher in the field of endocrinology who wishes to extend the opportunities for work either in this country or abroad.

The award will not exceed $2,500 annually and will be granted on the basis of proposals submitted by the applicant. Such applications should include estimated financial needs. The funds may be used for travel, maintenance and other expenses.

CITATIONS FOR THE 1961 AWARDS

The 1961 Koch Medal

Lawson Wilkins:

In affection and esteem, we hail you and herewith confer upon you the highest honor we have in our power to bestow: the Koch Medal of The Endocrine Society.

As a former President of this Society, you know from how deep within us this tribute comes. For in your long but seemingly unwearing years of endocrinological study and teaching you have reached us all.

None of us can fail to be informed and stimulated by your eminent studies. Whatever order and system the pediatric endocrinologist now has he owes to your original development of correlated clinical and biochemical analysis in this complex and, at times, mystifying field. Your characterization of the various patterns of thyroid deficiency in children opened the way to our current knowledge and management of this disabling, yet reversible disease process. Your incisive study of the disturbed physiology of the adrenogenital syndrome led you to a comprehension of the several varieties of this affliction which in turn has provided its victims with one of the most effective therapies known to our specialty. Your keen appreciation of fundamental biological relationships resting on a firm foundation of embryological and anatomical knowledge has enlightened our entire

view of the nature of the disorders of gonadal development and function which blight the body and spirit of so many unfortunate children.

And with all these monumental achievements you have brought to your patients the comfort and assurance that can only be generated by the kindly concern of one who is genuinely devoted to others. And this same benevolence and interest reflects itself in the fine array of that highly select and yet numerous band of younger men and women who have been privileged to call you their teacher. Yet all of us interested in endocrinology through your explicit and instructive writings have shared in this privilege.

Hence it is only fitting that we here collectively, through your own Endocrine Society, tender you this small, but to us precious indication of our great indebtedness to you and of our ever constant affection for you—Doctor Lawson Wilkins!

The 1961 Ciba Award

The 1961 recipient of the Ciba Award is Dr. Ernst Knobil, Assistant Professor of Physiology in the Harvard Medical School. The Endocrine Society takes pride in adding his name to the roster of distinguished men and women upon whom this award has been conferred.

Dr. Knobil was born in Berlin and reared in Paris, where he was schooled in the demanding French educational clime. Although he attended the Claude Bernard Lycee, it is not likely that this circumstance launched his career in experimental physiology in that it was not until sometime later that he learned about Claude Bernard! He was early inclined to the arts and once contemplated a career in architecture. A youthful fling at sculpturing in a New York art school yielded some objects for exhibit, but their present fate is obscure.

At age 15 Ernie entered the School of Agriculture at Cornell to major in animal husbandry. Art and architecture paled before the spectacle of rural America, which inspired him to indulge in the intricacies of wool growing, herd fattening and flock culling. That his perceptive powers were challenged is evident from the fact that he made the Livestock Judging Team and won a number of prizes.

After the sophomore year he was called to the military and assigned to the Information and Education Branch at Fort Dix as a specialist. He came to head the Indoctrination staff and with typical individual initiative he voluntarily taught French and German courses by night for officers going overseas and established a training course for information specialists. Contact with outside education came in the form of an assignment to arrange for the schooling of the children of camp personnel in neighboring public schools. Convincing local school boards to accept more students into schools already overcrowded and understaffed sharpened his persuasive powers.

After the war Dr. Knobil returned to Cornell, bent on a closer look at the sciences. His enthusiasm for the pure sciences was fired by distinguished scholars and teachers such as Sidney Asdell, Glen Salisbury and Samuel Leonard. It was Asdell's course "The Physiology of Reproduction" that settled the direction of his graduate work, which was done in zoology

with Dr. Leonard. Courses with J. B. Sumner and Donald Griffin among other campus notables deepened his scientific curiosity.

He gained his postdoctoral experience in research at Harvard with R. O. Greep and associates. With Greep he spearheaded the demonstration of the species specificity of growth hormone in primates and thereby provided the clue to its successful use in man. He has independently made important observations in the fields of hypophyseal, adrenal and reproductive physiology. Currently, as evidenced by his report at this meeting, he is exploring avenues in cellular biology looking to the mechanism of hormone action at the molecular level. More than a talented investigator, Dr. Knobil is a stimulating teacher and has regularly enjoyed the acclamation of students for his orderly and dynamic lectures. He is a Markle Scholar and a member of the Editorial Board of *Endocrinology* and the *American Journal of Physiology*. As Acting Editor of *Endocrinology* for one-half year he discharged the responsibilities of that office with skill and resourcefulness.

The fates of fortune have surrounded this young scholar. Within the month he will, at thirty-four years of age and as Professor and Chairman of the Department of Physiology, occupy the Mellon Chair in the School of Medicine at the University of Pittsburgh.

Dr. Knobil, the distinguished recognition which comes to you here as recipient of the 1961 Ciba Award is based on your outstanding achievements in science. It is in keeping with the important responsibilities in medical education and research which lie before you. You have the congratulations and well wishes of this Society.

The Ayerst Fellowship

Dr. John Maxwell McKenzie is the recipient of the Ayerst Fellowship for 1961, presented by The Endocrine Society in acknowledgement of his contributions to our understanding of the pathogenesis of Graves' Disease. Dr. McKenzie was born in Glasgow, Scotland, on November 13, 1927. His early schooling and University training were all in his native Scotland. He was Dux Medallist upon completing his secondary school training at Rothesday Academy. He received the M.B., Ch.B., from the University of St. Andrews, Dundee, in 1950. Dr. McKenzie's post-graduate training included: House Surgeon, House Physician, Maryfield Hospital, Dundee; Assistant, Department of Pharmacology and Therapeutics, University of St. Andrews; Registrar, Maryfield Hospital. It was during this early phase of his training that he came under the influence of Professor R. B. Hunter whose enthusiasm for research sparked in Max McKenzie the desire to win a place for himself in Academic Medicine. With this in view he joined Dr. E. B. Astwood's laboratory as a Research Fellow in Endocrinology, New England Center Hospital, and Assistant in Medicine, Tufts University Medical School. He returned to the Maryfield Hospital, Dundee, as a Registrar for a one year period and once more joined Dr. E. B. Astwood's department as a Research Associate in Medicine, New England Center Hospital and an Instructor in Medicine, Tufts University Medical School. It was during this time that his thesis for the degree of M.D. of the University of St. Andrews entitled "The Bioassay of Thyrotropin in Serum"

was accepted and awarded Honours and the University Gold Medal. In 1959 he joined the Endocrine-Metabolic Unit of the Department of Medicine and the McGill University Clinic, Royal Victoria Hospital, Montreal. At present he is in charge of the Thyroid Laboratory, a Lecturer in Medicine, McGill University and a Clinical Assistant in the Department of Medicine, Royal Victoria Hospital.

Dr. McKenzie's contributions to endocrinology have been in the field of thyroid physiology, particularly in the development of bio-assay techniques for T.S.H. and the "Thyroid activator" of hyperthyroidism. It is with the isolation and characterization of the latter factor that he is particularly concerned at the present time. The Ayerst Fellowship will allow him to spend time in the laboratories of Dr. C. J. O. Morris at the London Hospital, London, England, gaining further experience in techniques of separation of protein and polypeptide hormones.

Dr. McKenzie is a careful and able investigator, a competent physician, a quiet, amiable and humble colleague. He succeeds in infusing a unique enthusiasm into all those with which he comes in contact, an attribute which permits his laboratory to accomplish an impressive amount of productive work. The Awards Committee has every reason to predict that his future contributions will continue to reflect credit upon this Society.

Upjohn Scholars

The Upjohn Scholars for 1961 are Dr. and Mrs. Wilhelmi.

Dr. Jane Russell (Mrs. Wilhelmi) was born in Los Angeles. She received her B.A. and Ph.D. degrees from the University of California and spent a year of post-doctoral study at the Institute for Experimental Biology at Berkeley. In 1938 she moved to Yale to continue her work on the pituitary. It was while at Yale that she married Alfred Wilhelmi. In 1950 the Wilhelmis moved to Emory University where Dr. Russell holds the position of Associate Professor of Physiological Chemistry.

In recognition of her classic studies on the role of the adenohypophysis in carbohydrate and protein metabolism, Dr. Russell was given the Ciba Award of the Endocrine Society in 1945. She is a member of the National Science Board, a high honor indeed, for this body of scientists appointed by the President of the United States is charged with the responsibility of guiding the policies and operations of the National Science Foundation.

Dr. Alfred Wilhelmi was born in Lakewood, Ohio. Following receipt of his B.A. degree from Western Reserve University, he spent three years at Oxford University as a Rhodes Scholar. From Oxford he went to Yale, Department of Physiological Chemistry, where he rose to the rank of Associate Professor. In 1950 he became the Chairman and Professor, Department of Biochemistry, Emory University.

Dr. Alfred Wilhelmi has made notable contributions in a number of areas including creatine metabolism, parathyroid hormone, growth hormone and comparative endocrinology of pituitary hormones. As a member of the Endocrinology Panel of the N.I.H., he has made a most notable contribution to endocrinology by making available high purified pituitary hormones for metabolic studies. Many of his fellow endocrinologists are most grateful indeed.